Surface Recognition of a Protein Using Designed Transition Metal Complexes

Md. Abul Fazal, Bidhan C. Roy, Shuguang Sun, Sanku Mallik,* and Kenton R. Rodgers

Contribution from the Department of Chemistry, North Dakota State University, Fargo, North Dakota 58105

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Abstract: Each protein has a unique pattern of histidine residues on the surface. This paper describes the design, synthesis, and binding studies of transition metal complexes to target the surface histidine pattern of carbonic anhydrase (bovine erythrocyte). When the pattern of cupric ions on a complex matches the surface pattern of histidines of the protein, strong and selective binding can be achieved in aqueous buffer (pH = 7.0). The described method of protein recognition is applicable to proteins of known structures. With rapidly increasing number of solved protein structures, the method has wide applicability in purification, targeting, and sensing of proteins.

Strong and selective binding to a complex biomolecule in aqueous solution by synthetic receptors is an area of active research.[1] Molecules that bind to a particular protein with a high affinity have many potential applications including design of new and selective enzyme inhibitors,[2] design of stationary phases for chromatographic purification of proteins[3] and construction of protein sensors.[4] Recognition of an enzyme can be achieved by exploiting a structurally well-defined enzyme active site or a unique section of the protein's surface topology.

Each protein has a unique pattern of amino acid residues on the surface. It is this pattern that a surface receptor recognizes for binding and selectivity. Protein surface patterns can be recognized by polypeptides,[5] non-peptide charged moieties,[6] sugars,[7] etc. If the receptor binds on the surface of an enzyme close to the active site, this method can lead to efficient inhibitors[8a,b] or disruptors for protein−protein interactions.[8c,d] Combinatorial approaches to generate peptides and small molecules capable of binding to a protein have been demonstrated.[9] Linking two or more of these receptors improves the binding affinity and the selectivity by large increments.[10]

Another approach for recognizing the surface pattern of amino acids of a protein involves template polymerization.[11] The methodology has been performed on a surface[12] or using a gel.[13] In this approach, a pattern complementary to that of the protein template is created on the polymer.

We are interested in the recognition of peptides[14] and proteins[15] employing metal−ligand interactions. Various transition metal ions (e.g., Cu^{2+}, Ni^{2+}, Zn^{2+} etc.) bind to the imidazole side chains of surface exposed histidines of proteins.[16] This coordination interaction (M^{2+}−His) has been used for protein purification by immobilized metal affinity chromatography (IMAC),[17] protein targeting to a surface[18] and two-dimensional

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protein crystallization.[19] For these applications, the proteins are distinguished on the basis of their surface histidine contents.

The goal of this study is to use metal−ligand interactions to selectively bind to peptides and proteins by recognizing unique patterns of surface-exposed histidines. Herein, we report our results on the recognition of the enzyme, carbonic anhydrase from bovine erythrocytes (25 mM HEPES buffer, pH = 7.0, 25 °C). Recognition has been achieved with ligands that bind three cupric ions such that the metal centers can coordinate to multiple surface histidie residues simultaneously. The reported method of protein recognition is applicable to proteins of known structures. With rapidly increasing number of solved protein structures available from the data bank,[20] the method has wide applicability.

Metal−ligand (M−L) interactions offer several advantages in recognition compared to hydrogen bonding, ion-pair or other interactions. The M−L interactions are stronger in water[21] compared to other interactions mentioned; thus, a fewer number of simultaneous and complementary interactions can lead to tight and selective binding. The spectroscopic properties of the metal ions can be used to monitor the binding process and to obtain structural information about the resultant complex.[22] A variety of transition metal ions are available and are used by nature in biological systems for molecular recognition and catalysis.[23] This allows fine-tuning of each of the interactions and opens the possibility of catalysis following recognition [24] (not demonstrated in this study).

Results and Discussion

Design and Syntheses of the Metal Complexes. To demonstrate the proof-of-concept, carbonic anhydrase (CA, bovine erythrocyte) was taken as the target protein. CA has six histidines on the surface (1, 7, 12, 14, 61, and 93 determined from the X-ray structure[25] using the modeling software insight II and Discover, version 98.0, Molecular Stimulations Inc., Burlington, MA). The distances separating the histidines 1, 7, 14 (or 12) are 13 ± 2, 16 ± 2, and 17 ± 2 Å respectively. In the studies reported here, either His-14 or His-12 can participate in binding. In this study, no attempts were made to dissect these two binding modes as either one is consistent with the conclusions reached.

The reported inter-histidine distances were measured using the ϵ-N atom of the imadazole moiety (Figure 1). Histidines 61 and 93 are separated from the targeted histidine triad by a peptide loop. Calculations (using insight II and Discover) suggest that these two histidines are less exposed to solvent compared to histidines 1, 7, 14 (or 12, Supporting Information).

Three other proteins were used as controls (chicken egg albumin, horse skeletal muscle myoglobin, and chicken egg lysozyme). Chicken egg albumin (CEA) has six solvent-exposed histidines (22, 23, 329, 332, 363, 371). CEA has the same number of surface histidines as CA, but the pattern is different. Myoglobin (Mb) has seven histidines on the surface (36, 48, 81, 97, 113, 116, 119). Thus, Mb has a greater number of surface histidines than the target protein (CA) but with a different distribution. Lysozyme has only one histidine (15), and it is exposed on the surface. The histidine distributions of the control proteins are also depicted in Figure 1.

All of these proteins (target and the controls) are known to interact with transition metal ions and complexes through the surface-exposed histidines.[26] If recognition is based solely on the number of surface histidine residues (e.g., IMAC), then Mb is expected to have the highest affinity for the metal complexes; the target protein CA and CEA should exhibit similar binding strengths.

To bind to CA selectively, the tris-Cu^{2+} complex **2** (Figure 2) was designed. The presence of the (1,3,5-triphenyl)phenyl core makes the complex rigid. Iminodiacetic acid (IDA) was chosen as the ligand to chelate the cupric ions. IDA has strong affinity for Cu^{2+} ($K \approx 10^{12}$ M^{-1}),[27] and the resultant complex has been widely used in protein purification by IMAC.[17] This ensures that the complex will not be demetalated even at high protein concentrations. It is reported in the literature that IDA−Cu^{2+} complexes bind to proteins (pH = 7.0) primarily through the histidine residues on the protein surface.[28] Complexes **1**, **3**, **4**, **5**, and **6** (Figure 2) were designed as the controls for these studies.

The inter-copper distances were estimated by molecular modeling employing the software Spartan (version 5.2, Wave function Inc., Irvine, CA). The complexes were energy-minimized in the gas phase (employing Merck molecular mechanics force field) followed by systematic conformational searches to locate the lowest energy conformations. The distances between the Cu^{2+} ions were estimated to be ∼12 Å for **1**, ∼16 Å for **2**, ∼12−16 Å for **3**, ∼14−18 Å for **4**, and ∼8−12 Å for **5**.

Complex **1** was designed to position three cupric ions at a distance that does not match the inter-histidine distances of CA. Complexes **3** and **4** were designed to probe the role of rigidity versus flexibility in the recognition process. Complex **5** has five cupric ions, but the pattern is not complementary to histidine pattern of the target protein (CA). Complex **6** has one Cu^{2+} and was used as another control for these recognition studies.

The syntheses of **1**, **2**, **3**, **4**, and **5** are indicated in Scheme 1. Elemental analyses indicated that the complexes had the correct number of cupric ions. Determination of Cu^{2+} by UV−vis spectrometry (employing EDTA) also indicated the proper number of Cu^{2+} ions. The complexes were found to be stable in aqueous buffer solution (25 mM HEPES buffer, pH = 7.0, 25 °C) in air for more than a month.

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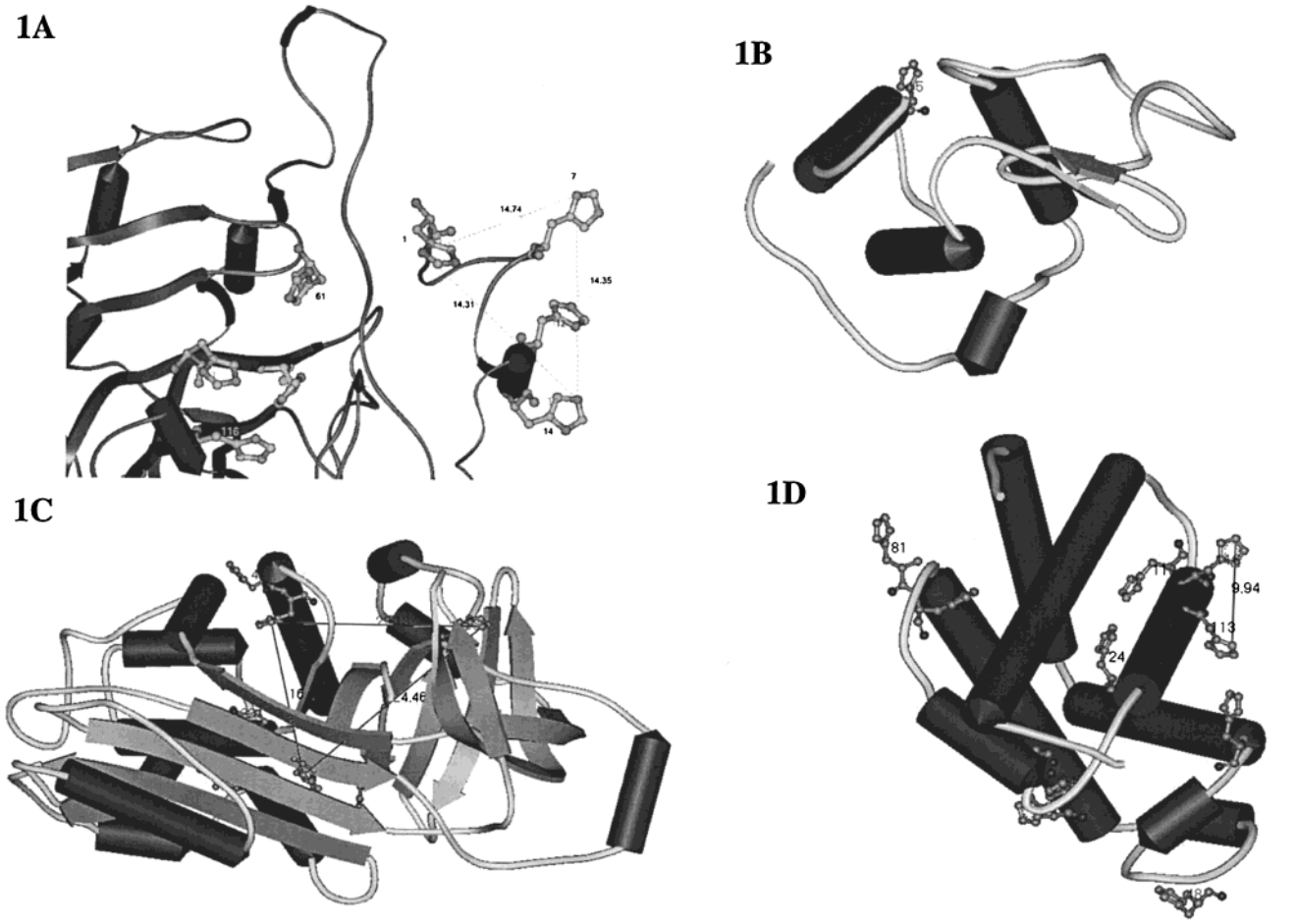

Figure 1. Histidine distributions of the target protein CA (1A) and the controls, lysozyme (1B), CEA (1C) and Mb (1D) used in this study. The solvent accessibility of the histidine residues are included in Supporting Information.

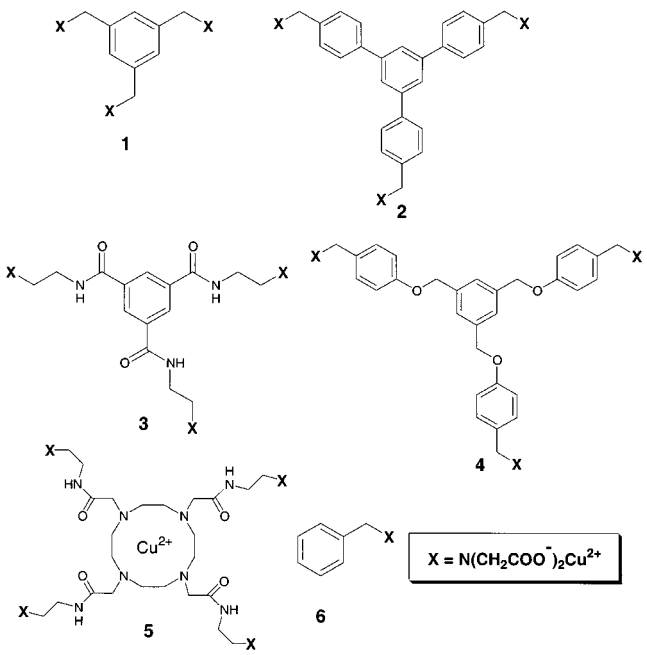

Figure 2. Structures of the metal complexes.

Binding Studies by Isothermal Titration Microcalorimetry. Binding studies were performed in HEPES buffer (25 mM, pH = 7.0, 25 °C) and were studied by isothermal titration microcalorimetry (ITC)[29] or by UV−vis spectrometry. In a typical ITC experiment, a solution of the protein (~100 μM) in the ITC cell was titrated with a solution of the metal complex. The heat of dilution of the metal complex was then separately determined by injection of a solution of the complex in buffer. The software provided by the manufacturer (*Bindworks*, v 3.0, Calorimetry Sciences Corporation, Provo, UT) was used to correct for the heat of dilution and subsequent data processing by nonlinear regression of the titration data. A multiple independent binding site model fits the data best. Other binding models (two sets of independent binding sites and cooperative binding) did not fit the titration data well. A typical titration curve (raw and processed data) is shown in Figure 3 (titration of CA with **2**). Results of ITC titration experiments are shown in Table 1. The data processing gave the stoichiometry (n), affinity (K), and the enthalpy (ΔH) of the interactions. The entropy values were calculated using the experimentally determined parameters (K, ΔH).

The control metal complex **6** (containing one cupric ion) showed weak affinity for the proteins tested ($K < 7500$ M^{-1}). Myoglobin bound more than more equivalents of **6**; with CEA, the affinity was weak, and reliable values were difficult to obtain. The control protein lysozyme (with one histidine on the surface) was found to have low affinity ($K < 5000$ M^{-1}) for all of the complexes used in this study.

Strong binding to the target protein (CA) was achieved by the distance-matched tris-Cu^{2+} complex **2** ($K = 299\,000 \pm 30\,000$ M^{-1}). When other metal complexes were used (distances

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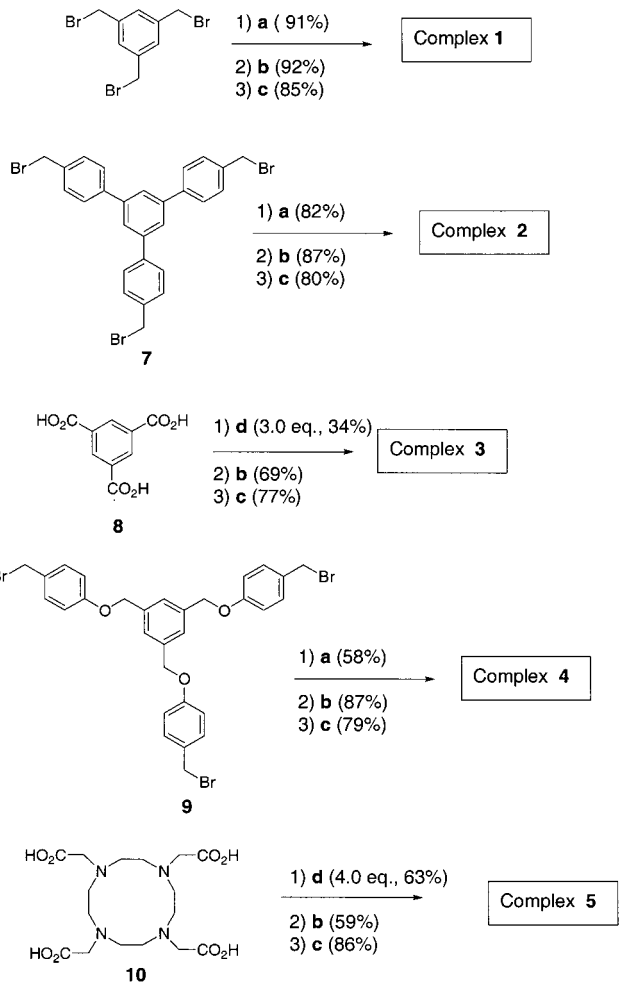
Scheme 1[a]



[a] Reagents and conditions: **a**: $HN(CH_2CO_2Et)_2$ (3 equiv), K_2CO_3 (10 equiv), CH_3CN. Sonication, 24 h. **b**: LiOH (9 equiv), MeOH−THF, 25 °C, 15 h then H_3O^+ (pH = 3.0). **c**: $CuCl_2 \cdot 2H_2O$ (3 equiv), MeOH−H_2O, 25 °C, 3 h. **d**: $H_2NCH_2CH_2N(CH_2CO_2Et)_2$ (**11**), Et_3N, BOP, CH_3CN.

not matched), lower affinities were observed. Similarly if the pattern of cupric ions was kept constant (i.e., complex **2**) and the histidine pattern on the protein surface was varied (i.e., proteins other than CA), binding constants were lower. A metal complex containing more number of cupric ions (**5**, inter-Cu^{2+} distances not matched with the inter-histidine distances of the targeted histidine triad) demonstrated weak affinities for all of the proteins tested. The binding constants reported in Table 1 are graphically illustrated in Figure 4.

Comparison of the binding constants indicated that complex **2** is very selective for CA compared to CEA (300:1); with myoglobin, the selectivity is moderate (20:1). For myoglobin, two of the cupric ions of **2** may simultaneously bind to two histidine residues on the protein surface (His 113 and 116 or 119, see the inter-histidine distances in Figure 1). This will lead to decreased selectivity of **2** in distinguishing CA from myoglobin. Lysozyme precipitated in the presence of the complex **2**; this prevented us from determining the binding constant using ITC. With the complex **1**, possibly the binding is more strained with CA ($\Delta H = -12.7$ kcal/mol for **1**; −15.1 kcal/mol for **2**). The flexible complexes **3**, **4**, and **5** showed less strain of binding compared to **1** and **2** but considerably more loss of entropy upon binding to CA. The three flexible complexes have similar distances among the cupric ions. The

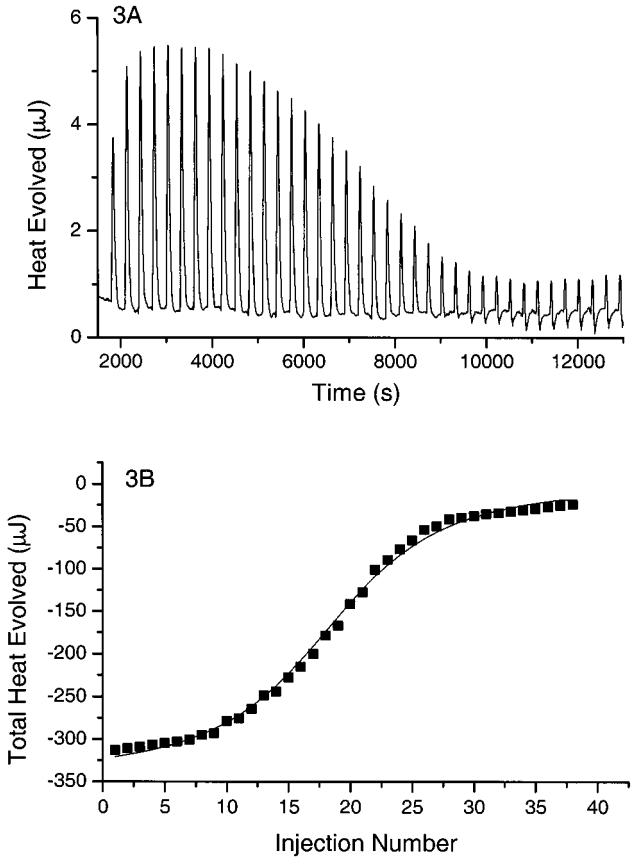


Figure 3. Raw titration curve (3A) and processed data (3B) for the titration of CA with the complex **2** (25 mM HEPES buffer, pH = 7.0, 25 °C, [CA] = 0.12 mM, [**2**] = 1.0 mM, 42 × 5 μL injections).

binding constants with CA were also similar. The flexible complex **3** had low entropic loss upon binding to CEA (we do not have any explanations yet for this observation), leading to a moderate affinity for the protein (K = 70 600 M^{-1}). If the organic ligands corresponding to the metal complexes were used, no binding was detected by ITC. This demonstrated the role of the metal ions in the binding process.

To demonstrate selective binding of **2** to the target protein, a mixture of CA and complex **6** ([CA] =100 μM; [**6**] =100 μM) was titrated with **2** ([**2**] = 0−500 μM). No change in the binding constant was observed for **2**. On the other hand, when a mixture of CA and **2** ([CA] = 100 μM; [**2**] = 100 μM) was titrated with **6** ([**6**]= 0−1 mM), no binding was detected. A mixture of the tested proteins (100 μM each) was titrated with complex **2** to obtain an unchanged affinity of **2** (280 000 M^{-1}). If CA was omitted from the protein mixture, very weak binding (K < 1000 M^{-1}) was detected. The selectivity reported here for complex **2** is distinctly different when compared to a random distribution of cupric ions (on chelating Sepharose fast flow with IDA−Cu^{2+} at pH = 7.0, 22 °C) either in equilibrium binding experiments or in chromatography).[30]

Effect of pH. The protein−ligand interactions are very sensitive to pH of the buffer as pH controls the protonation−deprotonation equilibria of both the protein and ligand molecules. The interactions between carbonic anhydrase and complex **2** were studied over the pH range 6.0−8.0. The lower affinity at pH = 6.0 (K = 199 000 ± 35 000 M^{-1}) is due to the partial protonation of the imidazole nitrogen (pK_a = 6.8). This protonation prevents the nitrogen atom of the imidazole groups

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Table 1. Stoichiometry (n), Binding Constant (K, M^{-1}), Enthalpy (ΔH, kcal mol^{-1}), Entropy ($T\Delta S$, kcal mol^{-1}) and Free Energy (ΔG, kcal mol^{-1}) for the Interactions of the Metal Complexes with the Proteins

proteins		control **6**	complex **1**	complex **2**	complex **3**	complex **4**	complex **5**
lysozyme	n	1.2 ± 0.3	$<10^3$	PPT	0.71 ± 0.04	PPT	$< 10^3$
	K	$(1.6 \pm 0.2) \times 10^3$			$(4.8 \pm 0.72) \times 10^3$		
	$-\Delta H$	7.0 ± 0.5			15.81 ± 1.36		
	$-\Delta G$	4.36			5.01		
	$-T\Delta S$	2.64			10.8		
carbonic anhydrase	n	0.90 ± 0.03	1.3 ± 0.01	0.94 ± 0.006	0.98 ± 0.03	0.83 ± 0.03	1.22 ± 0.03
	K	$(3.5 \pm 0.7) \times 10^3$	$(75 \pm 6) \times 10^3$	$(299 \pm 30) \times 10^3$	$(36.7 \pm 7.3) \times 10^3$	$(33.3 \pm 6.7) \times 10^3$	$(33.6 \pm 4.5) \times 10^3$
	$-\Delta H$	39.2 ± 2.0	12.7 ± 0.2	15.1 ± 0.3	23.62 ± 1.48	23.40 ± 2.0	20.88 ± 1.2
	$-\Delta G$	4.83	6.64	7.46	6.22	6.16	6.17
	$-T\Delta S$	34.37	6.06	7.65	17.4	17.24	14.71
chicken egg albumin	n	$<10^3$	1.6 ± 0.07	$<10^3$	1.55 ± 0.01	1.70 ± 0.02	0.90 ± 0.06
	K		$(16 \pm 2.5) \times 10^3$		$(76.5 \pm 7.0) \times 10^3$	$(32.0 \pm 3.4) \times 10^3$	$(18.4 \pm 3.3) \times 10^3$
	$-\Delta H$		20.9 ± 1.5		14.03 ± 0.26	6.97 ± 0.14	15.54 ± 1.8
	$-\Delta G$		5.73		6.65	6.14	5.81
	$-T\Delta S$		15.17		7.38	0.83	9.73
myoglobin	n	1.6 ± 0.02	0.74 ± 0.03	1.62 ± 0.03	1.00 ± 0.04	0.56 ± 0.02	0.53 ± 0.02
	K	$(7.2 \pm 0.6) \times 10^3$	$(49 \pm 6.2) \times 10^3$	$(20.4 \pm 5.2) \times 10^3$	$(8.2 \pm 0.7) \times 10^3$	$(3.5 \pm 0.1) \times 10^3$	$(12.3 \pm 1.7) \times 10^3$
	$-\Delta H$	4.2 ± 0.4	21.6 ± 1.3	16.62 ± 2.4	28.09 ± 2.0	81.19 ± 14.5	34.22 ± 5.8
	$-\Delta G$	5.25	6.39	5.87	5.33	4.83	5.57
	$-T\Delta S$	1.95	15.21	10.75	22.76	76.36	28.65

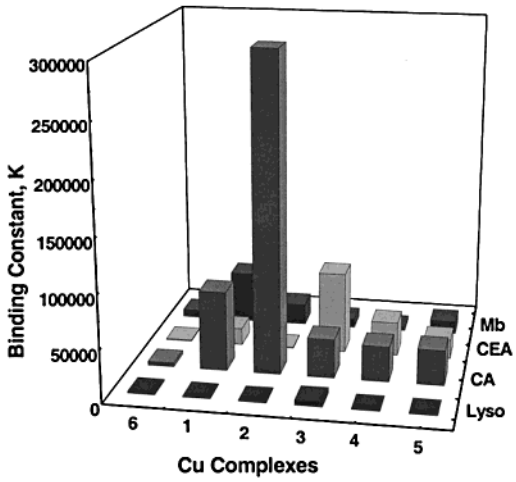

Figure 4. Binding constants of the metal complexes with the proteins.

to donate the lone pair of electrons to the Cu^{2+} ions.[31] On the other hand, at high pH (8.0), the IDA−Cu^{2+} complexes start forming insoluble precipitates and the binding affinity drops ($K = 97\,000 \pm 16\,000$ M^{-1}). In addition, at pH = 8.0 the net charge of IDA−Cu^{2+} complex is −1 which also reduces the affinity of the complex to accept the lone pair from imidazole group.[32]

Effect of Buffer Structure and Concentration. Three buffers (HEPES, MOPS, CHES) with different concentrations (25−100 mM) were used to study carbonic anhydrase−**2** interactions. Neither the buffer type nor the buffer concentration showed any significant effect on the binding affinity. Although protein−ligand interactions have been reported to be influenced by buffer types in some cases,[33] the reverse is also reported.[34]

Effect of Ionic Strength. The effects of ionic strength on the affinity of carbonic anhydrase and Cu^{2+} complex **2** were studied by varying NaCl concentration from 0.0 to 0.6 M at pH = 7.0 (25 mM HEPES buffer). The binding constant was found to decrease with increasing salt concentrations (Table 2). When the salt concentration was increased above 0.6 M, complex **2** was found to precipitate. This prevented us from studying the salt effect beyond the reported concentration range.

Table 2. Binding Parameters of CA with **2** as a Function of Salt Concentration

[NaCl] M	n	K_a (M^{-1})	$-\Delta H$ kcal mol^{-1}	$-\Delta G$ kcal mol^{-1}	$-\bar{T}\Delta S$ kcal mol^{-1}
0.00	0.94 ± 0.006	$299{,}400 \pm 30{,}200$	15.1 ± 0.32	7.46	7.65
0.10	1.06 ± 0.02	$232{,}600 \pm 12{,}200$	16.36 ± 0.72	7.31	9.05
0.20	0.98 ± 0.003	$215{,}800 \pm 14{,}300$	16.99 ± 1.03	7.27	9.72
0.30	0.96 ± 0.002	$125{,}400 \pm 8{,}500$	18.34 ± 0.82	6.95	11.39
0.60	1.21 ± 0.001	$78{,}600 \pm 10{,}200$	20.26 ± 0.43	6.67	13.59

The salt effects on immobilized Cu(II) with histidine and proteins have been studied extensively by various authors.[31,35] The interactions between solutes and immobilized Cu(II) consist of the formation of a coordination compound, the hydrophobic or van der Waals force, and electrostatic interactions.[31] In cases where donor−acceptor interaction is the primary force of binding mode, addition of NaCl at a high concentration would cause the free coordination sites to be occupied by anions. As a result the binding constant should be decreasing with increasing NaCl concentration. In addition, ionic strength also affects the protein surface through accumulation of anions and cations on it and results in a high repulsive force between the ligand and protein.

In our study, the change in binding constant as a function of salt concentration behaved well. In the studied concentration range of salt, the binding constant decreased steadily. The binding stoichiometry (n), enthalpy change (ΔH), and free energy change (ΔG) suggested that the same type of binding mechanism was in effect.

Binding Studies by UV−Vis Spectrometry. The binding of CA with the metal complexes can be followed by UV−vis spectrometry. Upon addition of CA to a solution of **2**, the absorption maximum of the Cu^{2+} ions progressively shifted from 727 to 660 nm ([**2**] = 0.5 mM; [CA] = 0.8 mM; 25 mM HEPES buffer, 25 °C, pH = 7.0; 10 × 100 μL additions). Further addition of the protein did not change to the absorption maximum. This shift indicates the coordination of one imidazole

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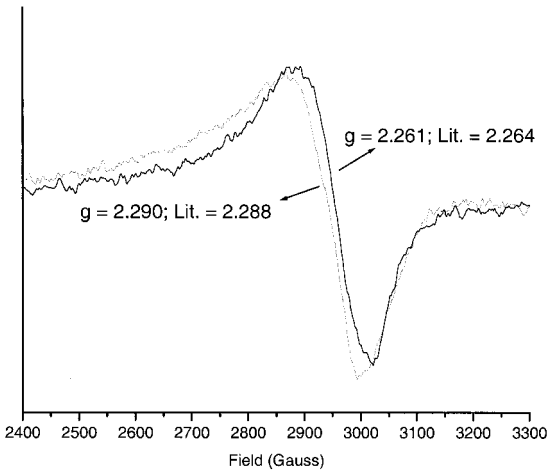

Figure 5. EPR spectra of complex **2** in absence and presence of the protein CA.

group (of histidine) to each cupric ions of the complex **2**.[36] The coordination of a second imidazole to the cupric ions is characterized by a further large blue-shift of the absorption maximum (628 nm).[36] Analysis of the resultant titration curves[37] (data not shown) corroborated the ITC results (for stoichiometry and binding constants).

For these titration experiments, an isobestic point was observed in the UV−vis spectra at 590 nm (Supporting Information). Another isobestic point started to form at 400 nm; however, when the molar ratio (complex **2**:CA) was close to 2, this isobestic point was lost. This suggests that at higher concentrations of **2**, CA may not be forming 1:1 complex with **2**.

Binding Studies by EPR Spectroscopy. The role of Cu^{2+}−histidine interactions in these studies was also demonstrated by EPR spectroscopy in room-temperature solutions (9.44 GHz, [CA] = 1.2 mM; [**2**] = 600 μM, 25 °C). Upon addition of the solution of CA to a solution of the metal complex **2**, the apparent $g_\perp$ value of Cu^{2+} ions decreased from 2.29 to 2.26 (Figure 5). These values are consistent with the coordination of an imidazole moiety to the cupric ions and displacement of a bound water molecule from the coordination sphere of IDA−Cu^{2+}.[38]

Circular Dichroism (CD) Studies. Circular dichroism spectroscopy has been applied to monitor the secondary and tertiary structural changes in many protein−ligand,[39] protein−lipid,[40] peptide−ligand[41] interactions. The far-UV circular dichroism spectrum (the peptide region) of a globular protein primarily reflects secondary structure while near-UV circular dichroism spectra (the aromatic region) primarily reflects its tertiary structure.[42]

Circular dichroism studies (0.3 mM CA, 0.6 mM metal complex **2**, 25 mM phosphate buffer, pH = 7.0, 25 °C) were conducted to detect any structural changes to CA upon binding to the metal complex (Supporting Information). Extensive purging with nitrogen was used to remove dissolved oxygen from the solutions. In presence of **2**, the CD spectrum of CA was found to change in the region 220−200 nm. The spectra (normalized to residue molar elipticity) were analyzed with the software DICROPROT[43] by self-consistent method to get structural information from the spectral changes.

Carbonic anhydrase, in the native form, consists of 7.7% α-helices and 25.8% β-sheets (calculated by the Structure Explorer module of the Protein Data Bank[20]). Analysis of the CD spectra of CA by DICROPROT predicted that the enzyme has 7.2% α-helices and 26.5% β-sheets. Analysis of the CD spectra of CA.**2** indicated that the content of α-helices was 8.4% and the amount of β-sheet was 31.8%.

The histidine triad targeted by complex **2** is located near the N-terminus of the protein. The N-terminal region of the protein has no ordered secondary structure; starting from residue 16, a short α-helical region starts (analyzed by insight II). It is possible that the random coil containing the histidines (1 and 7) may organize into a helical structure upon binding to complex **2**. The binding energy of **2** with CA is sufficient to compensate for the energy requirement of this structural change. We do not have any hypothesis yet on the increase of β-sheet contents of CA upon binding to **2**. The enzyme was found to retain its full activity in the presence of two equivalents of complex **2**.

Electrophoresis Studies. Many transition metal (e.g., Co^{3+}, Zn^{2+}, Ni^{2+}, Cu^{2+}) complexes have been reported to cleave amide bonds in peptides[44] and proteins[45] under mild conditions (pH = 7.0) through hydrolysis. In the literature, SDS-PAGE has been used to monitor the hydrolysis of protein in the presence of macrocyclic Cu^{2+} complexes.[45] Results from SDS-PAGE (data not shown) indicated no cleavage products for the protein (CA) in the presence of excess **2** (CA:**2** = 1:2, pH = 7.0).

Conclusions

In conclusion, we have demonstrated that a protein can be targeted with good selectivity by exploiting its surface histidine pattern using complementary transition metal complexes. The cupric ions coordinate to histidines exposed on the surface of the protein being targeted. The protein preserved its native structure in the presence of the metal complexes. Targeting of other proteins using this methodology is being carried out and the results will be reported in future.

Experimental Section

Syntheses of the Complexes. The ^{1}H and ^{13}C NMR spectra were recorded using 300, 400, and 500 MHz spectrometer with TMS as the internal standard. Elemental analyses were carried out by the in-house material characterization laboratory. Commercially available reagents were used as supplied unless stated otherwise. The organic extracts were dried over anhydrous Na_2SO_4 and concentrated in vacuo. TLC was performed with Absorsil Plus 1P, 20 × 20 cm plates (Alltech Associates, Inc.). Chromatography plates were visualized either with UV light or in an iodine chamber.

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Complex 1. 1,3,5-Tri-(bromomethyl)-benzene (3.0 g, 8.40 mmol) and diethyliminodiacetate (4.76 g, 25.18 mmol) were dissolved in dry CH_3CN, and anhydrous K_2CO_3 (14.11 g, 100.00 mmol) was added. The resulting reaction mixture was sonicated (125 W bath sonicator) for 12 h at room temperature. The solids were filtered off, and solvent was removed under vacuo. The crude yellowish oily liquid was purified by silica gel column chromatography with 1% methanol in $CHCl_3$ (R_f = 0.2) to afford the pure ester. Yield: 5.22 g (91%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.15−1.24 (m, 18H), 3.45 (s, 12H), 3.84 (s, 6H), 4.01−4.18 (m, 12H), 7.24 (s, 3H).

The ester (2.00 g, 2.94 mmol) was dissolved in 30 mL of MeOH−THF (2:1), and the solution of $LiOH\cdot H_2O$ (1.11 g, 26.43 mmol) in MeOH (10 mL) was added. The mixture was stirred at room temperature for 12 h, after which it was acidified with concentrated HCl (pH = 3.0). The white solid was filtered and washed with THF−MeOH mixture (4:1). This solid was again dissolved in 3 mL of water, THF was then added until two layers partitioned. The upper layer was removed carefully, and the bottom layer was dried under vacuo to afford the acid as a white solid. Yield: 1.39 g (92%). ^{1}H NMR (500 MHz, D_2O) δ 3.84 (s, 12H), 4.55 (s, 6H), 7.76 (s, 3H). ^{13}C NMR (125 MHz, D_2O) δ 58.86, 60.70, 133.84, 138.49, 172.54.

To a solution of the acid (0.65, 1.26 mmol) in water (10 mL) was added 0.65 g (3.80 mmol) of $CuCl_2\cdot 2H_2O$ in MeOH (15 mL). The stirring was continued at room temperature for 4 h. The blue solid was filtered and washed with MeOH. Yield: 85%. Anal. Calcd. for $C_{21}H_{21}Cu_3N_3O_{12}\cdot 3H_2O$: C, 33.54; H, 3.62; N, 5.59. Found: C, 33.53; H, 3.59; N, 5.54.

Complex 2. A mixture of **7**[46] (1.40 g, 2.07 mmol), diethyliminodicaetate (1.17 g, 6.21 mmol), and anhydrous K_2CO_3 (2.5 g, 17.86 mmol) in MeCN was refluxed for 12 h and then sonicated for 12 h in a bath sonicator (125 W). The solids were filtered off, and solvent was removed under vacuo. A viscous oily yellowish liquid was obtained; this was purified by column chromatography (silica gel, 2% MeOH in $CHCl_3$, R_f = 0.4) to afford the pure ester as a viscous oil. Yield: 1.70 g (82%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.27 (t, 18H, J = 7.0 Hz), 3.58 (s, 12H), 3.78 (s, 6H), 4.18 (q, 12H, J = 7.0 Hz), 7.48 (d, 6H, J = 8.0 Hz), 7.63 (d, 6H, J = 8.0 Hz), 7.75 (s, 3H).

The ester (0.94 g, 0.94 mmol) was hydrolyzed with monohydrated LiOH (0.35 g, 8.33 mmol) in MeOH−THF (10/5 mL). The mixture was stirred at room temperature for 12 h, after which it was acidified with concentrated HCl (pH = 3.0). The white solid was filtered and washed with THF−MeOH mixture (4:1). This solid was again dissolved in 3 mL of water, THF was then added until two layers partitioned. Upper layer was removed carefully, and bottom layer was dried under vacuo to afford the acid as a white solid. Yield: 0.68 g (87%). ^{1}H NMR (270 MHz, D_2O) δ 3.13 (s, 12H), 3.76 (s, 6H), 7.45 (d, 6H, J = 8.1 Hz), 7.71 (d, 6H, J = 8.0 Hz), 7.84 (s, 3H). ^{13}C NMR (125 MHz, D_2O−NaOD) δ 59.8, 60.4, 126.9, 129.6, 133.1, 139.3, 141.8, 144.1, 182.1.

To a suspension of the acid (0.50 g, 0.60 mmol) in water/MeOH (6/10 mL) was added 1 N solution of NaOH solution until the solid completely dissolved (pH ≈ 7.0). A solution of $CuCl_2\cdot 2H_2O$ (0.30 g, 1.80 mmol) in MeOH was then added and stirred for 4 h at room temperature. The blue solid was filtered and washed with MeOH. Yield: 0.56 g (80%). Anal. Calcd. for $C_{39}H_{33}Cu_3N_3O_{12}\cdot 3H_2O$: C, 49.60; H, 3.74; N, 4.45: Found: C, 49.68, H, 3.90, N, 4.52.

Complex 3. To a solution of 1,3,5-benzenetricarboxylic acid (0.44 g, 2.09 mmol) in CH_3CN (40 mL), was added Et_3N (4 mL, 29.33 mmol) followed by the addition of BOP reagent (2.8 g, 6.32 mmol) and **11**·2TFA[14] (2.88 g, 6.26 mmol) in CH_3CN (20 mL). Stirring was continued at room temperature for 24 h. The reaction was quenched with saturated aqueous solution of NaCl. The mixture was then extracted with ethyl acetate, and the extract was successively washed with 4% aqueous citric acid, water, 4% aqueous $NaHCO_3$, and water. The organic layer was dried over anhydrous Na_2SO_4, and solvent was evaporated under vacuo to provide the crude amide ester. Purification was achieved by silica gel column chromatography with 4% MeOH in $CHCl_3$ as the eluant (R_f = 0.2) to afford the pure product as an oily viscous liquid. Yield: 0. 60 g (34%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.23 (t, 18H, J = 7.1 Hz), 2.94−3.00 (m, 6H), 3.46−3.54 (m, 6H), 3.55 (s, 12H), 4.14−4.19 (m, 12H), 8.05 (bs, 1H), 8.13 (bs, 2H), 8.65 (s, 3H).

This ester was hydrolyzed (0.54 g, 0.63 mmol) using $LiOH\cdot H_2O$ (0.32 g, 7.6 mmol) in a mixture of MeOH−THF (10/5 mL). The workup procedure was the same as the hydrolysis reactions described before. Yield: 0.32 g (69%). ^{1}H NMR (400 MHz, D_2O−NaOD) δ 2.79 (t, 6H, J = 6.6 Hz), 3.21 (s, 12H), 3.44 (t, 6H, J = 6.6 Hz), 8.28 (s, 3H). ^{13}C NMR (125 MHz, D_2O) δ 38.1, 58.6, 59.3, 132.3, 137.0, 172.4, 172.8.

The acid obtained in the previous step (0.18 g, 0.26 mmol) was dissolved in water (5 mL) by adding 4 drops of 1 N NaOH solution. The solution of $CuCl_2\cdot 2H_2O$ (0.14 g, 0.82 mmol) in MeOH (7 mL) was added dropwise, and the reaction mixture was stirred for 4 h at room temperature. The blue solid was filtered and washed with MeOH. Yield: 0.2 g (77%). Anal. Calcd. for $C_{27}H_{30}Cu_3N_6O_{15}\cdot 3H_2O$: C, 35.12; H, 3.93; N, 9.10. Found: C, 35.23, H, 3.81; N, 9.35.

Tris-bromide 9. A mixture of the 1,3,5-tris(bromomethyl)benzene (2.78 g, 7.79 mmol), 4-hydroxylbenzyl alcohol (2.90 g, 23.37 mmol), potassium carbonate (4.14 g, 30 mmol), and 18-Crown-6 (0.53 g, 2 mmol) in dry acetonitrile (100 mL) was refluxed under nitrogen for 4 days with vigorous stirring. The mixture was then cooled and evaporated to dryness under reduced pressure. Water (150 mL) was added to dissolve inorganic salts, and the white solid, after being filtered, was washed with 5% Na_2CO_3, water, and ether, respectively. The product was taken to the next step without further purification. Yield: 3.5 g (92%); mp 42−44 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.48 (s, 3H), 7.23 (d, 6H, J = 8.1 Hz), 6.98 (d, 6H, J = 8.1 Hz), 5.12 (s, 6H), 5.07 (t, 3H, J = 6.1 Hz, OH), 4.43 (d, 6H, J = 6.1 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 157.2, 137.7, 134.9, 127.9, 126.1, 114.4, 69.0, 62.5.

A suspension of the above compound (1.0 g, 2.05 mmol) in ether (75 mL, dried by $LiAlH_4$) was cooled to 0 °C, and a solution of PBr_3 (0.86 g, 3.2 mmol) in anhydrous ether (25 mL) was added slowly with stirring; the ice bath was then removed, and it was refluxed for 14 h. The mixture was cooled down, and the pale yellow solid was collected, successively washed with 2% HCl, water, $NaHCO_3$, and water, dried in air, and afforded 1.0 g (72%) of tris-bromide: mp 80−82 °C. ^{1}H NMR ($CDCl_3$) δ 7.44 (s, 3H), 7.32 (d, 6H, J = 8.5 Hz), 6.92 (d, 6H, J = 8.5 Hz), 5.09 (s, 6H), 4.50 (s, 6H); ^{13}C NMR ($CDCl_3$) δ 158.7, 137.8, 130.6, 130.5, 126.0, 115.2, 69.8, 33.9.

Complex 4. A mixture of **9** (0.3 g, 0.44 mmol), diethyl iminodiacetate (0.25 g, 1.23 mmol), and K_2CO_3 was taken in CH_3CN (25 mL) and sonicated in a bath sonicator (125 W) for 40 h. The workup procedure was the same as that described before. The resulting crude oily yellowish liquid was purified by silica gel column chromatography with 2% MeOH in $CHCl_3$ (R_f = 0.7). Yield: 58%. ^{1}H NMR (270 MHz, $CDCl_3$) δ 1.25 (t, 18H, J = 7.1 Hz), 3.51 (s, 12H), 3.83 (s, 6H), 4.15 (q, 12H, J = 7.1 Hz), 5.06 (s, 6H), 6.91 (d, 6H, J = 8.5 Hz), 7.28 (d, 6H, J = 8.5 Hz), 7.46 (s, 3H).

The ester (0.24 g, 0.24 mmol) was hydrolyzed with $LiOH\cdot H_2O$ (30 mg, 2.14 mmol) in MeOH−THF (5/5 mL) as described for other compounds. The acid was obtained as a yellowish solid (0.15 g, 87%). ^{1}H NMR (400 MHz, D_2O) δ 3.46 (s, 12H), 4.07 (s, 6H), 4.99 (s, 6H), 6.93 (d, 6H, J = 8.1 Hz), 7.28 (d, 8H, J = 8.1 Hz), 7.38 (s, 3H). ^{13}C NMR (125 MHz, D_2O) δ 58.1, 60.2, 70.5, 118.0, 124.3, 128.8, 135.2, 139.8, 161.8, 172.5.

A solution of $CuCl_2\cdot 2H_2O$ (0.07 g, 0.42 mmol) in MeOH (5 mL) was added to the solution of above acid (0.12 g, 0.14 mmol) in water (5 mL) at room temperature. The workup procedure was the same as that for complex **1**. Yield: 0.12 g (79%). Anal. Calcd. for $C_{42}H_{39}Cu_3N_3O_{15}\cdot 3H_2O$: C, 47.12; H, 4.24; N,3.93. Found: C, 47.34; H, 4.41; N, 3.73.

Complex 5. Tetraacid **10**[47] (0.54 g, 1.34 mmol) was dissolved in DMF−$CHCl_3$ (40 mL, 1:1) mixture in the presence of Et_3N (1.5 mL, 10.78 mmol) followed by the addition of **11** (1.68 g, 5.35 mmol) and BOP reagent (2.30 g, 5.35 mmol). The resultant mixture was stirred for 30 h at room temperature. The workup procedure was the same as that described complex **3**. The crude product was purified by silica gel column chromatography (R_f = 0.4 with 10% MeOH in $CHCl_3$). Yield: 1.06 g (63%), mp 38−40 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.27 (t,

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24H, $J = 7.0$ Hz), 2.31−2.49 (m, 8H), 2.81−2.88 (m, 8H), 3.15−3.26 (m, 16H), 3.53 (bs, 4H), 3.71−3.76 (m, 4H), 4.15 (q, 16H, $J = 7.0$ Hz), 7.40 (bs, 2H), 8.08 (s, 2H).

The ester (0.28 g, 0.22 mmol) was hydrolyzed with $LiOH \cdot H_2O$ (0.14 g, 0.33 mmol) in MeOH−THF solvent (5/5 mL) as described previously. Yield: 0.18 g, 59%. ^{1}H NMR (500 MHz, 60 °C, D_2O) δ 3.42−3.60 (m, 4H), 3.64−3.75 (m, 4H), 3.76−3.90 (m, 16H), 3.92−4.12 (m, 16H), 4.16−4.22 (s, 16H). ^{13}C NMR (125 MHz, D_2O) δ 37.4, 39.9, 50.9, 54.8, 57.2, 57.4, 57.8, 58.0, 58.2, 59.5, 59.6, 59.7, 60.7,-172.72, 172.74, 172.56, 171.78, 172.80.

The acid (0.30 g, 0.28 mmol) was dissolved in 10 mL of H_2O (the insoluble part was filtered off). The solution of $CuCl_2 \cdot 2H_2O$ (0.05 g, 0.29 mmol) in MeOH (7 mL) was added dropwise and stirred at room temperature for 3 h. The solvent was removed in vacuo. MeOH−H_2O (10 mL, 5:2) was added and stirred another 0.5 h. The solid was filtered and washed with MeOH. Yield: 0.32 g (86%). Anal. Calcd. for $C_{40}H_{68}Cu_5N_{12}O_{20} \cdot 2Cl^- \cdot 5H_2O$: C, 31.86; H, 4.68; N, 11.14. Found: 31.96; H, 4.31; N, 10.90.

Determination of Binding Constants. Calorimetric studies were performed with Isothermal Titration Calorimeter (ITC-4200, Calorimetry Sciences Corporation, Provo, UT) linked to a personal computer for data acquisition and analysis. An external water bath (NESLAB, RTE-111) 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. Hamilton syringes were used to load the sample solution (cell volume = 1.32 mL). A CENTRA-CL2 unit was used for centrifugation. Absorption spectra were recorded using a Ultroscope-4000 (Pharmacia Biotech) UV/visible spectrophotometer. Quartz cuvettes of 10 mm path length were used for all routine studies. The temperature effect on UV−visible spectra was studied using a Beckmann-60 unit fitted with a constant temperature accessory. DSC experiments were carried out with a Perkin-Elmer (DSC 7) unit and the sample cells provided by the manufacturer (KIT No. 0219−0041) were used. For EPR study a Varian model E-4 EPR spectrometer was used. Spectropolarimeter from Japan Spectroscopic Co. Ltd. (JASCO, J-810) was employed for CD measurements. Mini PROTEAN II from Bio-Rad was used for electrophoresis studies. Analytical balance (Ohaus AP 250D) was used for weighing samples (accuracy = 0.1 mg).

Buffers. Buffers were prepared by adjusting the pH (7.0 ± 0.01) at 25 °C with 1 N NaOH. Sodium azide (1%, W/V) was added as the preservative. After passing through a 0.22 μm cellulose membrane (Stericup, Millipore), the final solution was stored at 4 °C. Dilute HCl and NaOH solutions were used for further adjustment in pH (6.0−8.0). No consideration was made to take the temperature effect into account.

Isothermal Titration Calorimetry (ITC). Calorimetric measurements were conducted in aqueous solution (25−100 mM, HEPES, MOPS, CHES; pH = 6.0−8.0). Protein samples (0.075−1.5 mM) were routinely centrifuged prior to the titration and were examined for precipitates, if any, after the titration. No precipitate was observed even at the highest working temperature and ionic strength conditions. The final concentrations of the protein solutions were determined by UV−visible spectrophotometry at 280 nm. Similarly Cu^{2+} complexes were followed at 720−727 nm by UV−vis spectrophotometry. All solutions were thoroughly degassed by stirring under reduced pressure before use. The protein solution was placed in the ITC cell for protein−metal complex study. The metal complex of choice was loaded into the syringe. For all experiments the freshly prepared protein and Cu^{2+} complex solutions were used. An equilibration period of 30−45 min was used before starting the experiment.

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 metal complex solution into the reaction cell containing only the sample buffer. For every experiment the heat of dilution of the ligand was measured and subtracted from the sample titration values before data processing.

The temperature-dependent and ionic strength-dependent binding studies were conducted after the instrument had been equilibrated overnight at the required temperature or salt concentration. For all the experiments, the quantity $c = K \times [M]$, where [M] is the initial macromolecule concentration and K is association constant, was in the range of $2 < c < 200$ as required for isothermal microcalorimetry.

UV−Visible Spectroscopy. Solutions of CA (0.8 mM) and Cu^{2+} complexes (0.5 mM) were prepared in 25 mM HEPES buffer (pH = 7.0). For routine analysis at room temperature (23 °C), the absorption spectra were recorded in the 200−800 nm range against the blank (HEPES buffer). Titration studies were conducted by adding aliquots of CA to the cell containing a known initial concentration of **2**. To make solutions with a fixed mole ratio (**2**:CA), appropriate volumes of prepared solutions were mixed. All measurements were made after sample equilibration for at least 15 min.

Electron Paramagnetic Resonance (EPR). Solutions of CA and Cu^{2+} complex were made separately in HEPES buffer (25 mM, pH = 7.0). The solutions were checked for any precipitation and subjected to centrifugation if any precipitation was observed. The final concentrations of the solutions ([CA] = 1 mM; [**2**] = 0.5 mM) were determined by UV−vis spectrophotometry. The CA·**2** complex was studied by mixing the respective solutions in required proportion to get the protein in excess (CA:**2** = 2:1). All data were under the following conditions: frequency = 9.442 GHz; window width = 2000 G; amplifier gain = 10^4; modulation amplitude = 20 G; rate = 8.3 G/s; T = 25 °C. The reported spectra were obtained by averaging four individual scans.

Circular Dichroism (CD). Far-UV (190−260 nm) circular dichroism spectra were acquired for CA and complex **2** separately and in mixture (phosphate buffer, 25 mm, pH = 7.0, 25 °C) under continuous nitrogen purging. The protein and Cu^{2+} complex (**2**) concentrations were 0.3 and 0.6 mM, respectively. The protein−Cu complex mixture had a molar ratio of 1:2 (CA:**2**). All spectra were recorded using quartz cells with 1 mm path length. All spectra were corrected for the appropriate background. The final spectrum of each sample was an average of eight scans. The sample temperature was controlled at 23 ± 0.2 °C throughout the experiments.

Electrophoresis (SDS-PAGE). CA (0.1 mM) and CA·**2** complex (CA:**2** = 1:2) solutions were prepared in HEPES buffer (25 mM, pH = 7.0). The samples (10 μL/well) were then analyzed by electrophoresis through a 15% polyacrylamide gel with a running buffer (25 mM TRIS, 92 mM glycine, 0.1% SDS) of pH 8.3. The operating voltage was kept constant at 200 V. At the completion of the run (30−40 min), the gel was stained with a dye (0.1% Commassie Blue R-250, 45% CH_3OH, 45% H_2O, and 10% glacial acetic acid) for 20−25 min. Destaining was achieved by washing the gels repeatedly with freshly prepared destain solution (10% CH_3OH, 10% glacial acetic acid, 80% H_2O). After drying overnight, the gels were scanned to collect the final images.

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Supporting Information Available: Solvent accessibility surfaces of the histidines shown in Figure 1, ITC titration plots (raw and processed data) for the results reported in Table 1, UV−vis titration curve for CA with complex **2** and CD spectra of CA.**2** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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