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Immobilized Iminodiacetic Acid Metal Peptide Complexes. Identification of Chelating Peptide Purification Handles for Recombinant Proteins

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The chromatographic behavior of peptides containing histidine, aspartic acid, and lysine on Fe(III), Co(II), Ni(II), Cu(II), and Zn(II) Sephadex G25 iminodiacetic acid immobilized metal ion affinity columns was studied to identify peptides with a high affinity for immobilized metal ions. It is postulated that a unique chelating peptide (CP) can be attached to the N terminus of a protein, which allows that CP protein to be purified by using immobilized metal ion affinity chromatography (IMAC). None of the 49 peptides were retained strongly on Fe(II) and Zn(II) IMAC columns under the conditions used. The elution volumes of 21 lysine-containing peptides showed they were retarded to varying degrees on Ni(II) and Cu(II) IMAC columns. Histidine-containing di- and tripeptides were retained strongly, and many required a low-pH environment for elution. The lower the elution pH, or pH value of the peptide peak that was eluted during a pH gradient from pH 7.5 to pH 4.3, the higher the affinity of that peptide for the immobilized metal ion. Three histidine-containing peptides, His-trp, His-Tyr-NH_2, and His-Gly-His, out of 21 examined had unusually high affinities for Co(II), Ni(II), and Cu(II) IMAC columns. Possible structural features responsible for peptide binding to immobilized Cu(II) and Ni(II) are discussed. The metal ion binding capacities of commercially available iminodiacetic acid chelating gels were determined and found to follow the expected Irving–Williams order, as did the majority of peptides studied.

Introduction

Ligand-exchange chromatography[1] was introduced as an affinity purification technique for proteins using hydrophilic supports in 1975 by Porath[2] and was renamed immobilized metal ion affinity chromatograph or IMAC.[3] This affinity chromatography method takes advantage of transition-metal interactions with biological molecules, such as proteins and nucleic acids. Transition-metal ions are immobilized on Sepharose or Sephadex derivatized with iminodiacetic acid (IDA) chelating groups and allowed to interact with proteins in the mobile phase. Only those proteins with a high affinity for the particular immobilized metal ion are chemically adsorbed by forming coordinate-covalent bonds with unoccupied coordination sites on the metal. The adsorbed protein is washed free of other proteins in the sample and eluted by either lowering the pH of the mobile phase or introducing a displacing ligand in the buffer.

IMAC is a promising separation and purification method as demonstrated by its utility in purifying a number of proteins.[4-7] Unlike other affinity chromatography methods it is not yet possible to predict which proteins will bind a particular immobilized metal. Consequently IMAC is typically used to separate proteins into groups. Elevating IMAC from a group fractionation method to a method whereby individual proteins can be purified will require IMAC to become more predictable. Chromatographic predictability however requires a greater understanding of protein interactions with immobilized transition metals than presently exists. Sulkowski and co-workers have explored the surface topography of potential coordinating residues in various interferons and several model proteins with IMAC.[8] Porath and co-workers have studied the interaction of a few oligopeptides and all standard amino acids with IMAC columns in attempts to elucidate the factors responsible for protein binding.[9,10] Certain amino acid residues such as histidine, cysteine, and tryptophan have been implicated as participating in binding immobilized metal ions,[2] but predicting their surface exposure and accessibility for binding is unfeasible.

We examined the chromatographic behavior of a series of peptides containing lysine, aspartic acid, or histidine on Fe(III), Co(II), Ni(II), Cu(II), and Zn(II) IMAC columns with the intent of defining the binding requirements protein ligands have for immobilized transition metals and using that information to make IMAC predictable. Identifying a peptide with a high affinity for immobilized metal ions was the first step in testing our hypothesis that a highly specific metal-chelating peptide, CP, attached to the N terminus of a protein either chemically or biosynthetically, can be used to purify that proteins by using IMAC.[11] We propose that the ability of a dipeptide to bind immobilized metal ions can be transferred to a large polypeptide by simply attaching a peptide with a high affinity for metal ions to the N terminus of that polypeptide. This strategy, chelating peptide IMAC, or CP-IMAC, has widespread potential applications for proteins synthesized by recombinant DNA methods since the CP nucleotide sequence can be ligated to the exon coding for the desired protein so as to express a chimeric CP-protein product in a host organism, such as *Escherichia coli*. Such a purification strategy would increase the production rate of biosynthetic proteins, whether they be intended for pharmaceutical applications or use in site-directed mutagenesis studies where large numbers of pure protein analogues are needed to establish structure–function relationships.

Experimental Procedures

Materials. Peptides purchased from Sigma Chemical Co. or Chemalog were used as received. Amino acid analysis of all histidine-containing peptides confirmed their compositions. All other chemicals were reagent grade and were used without further purification. Distilled deionized water or Milli-Q filtered water was used throughout. The metal chelating resins used were from Pierce (immobilized iminodiacetic acid I and II) and Pharmacia (chelating Sepharose 6B).

Preparation of IMAC Columns. The following procedure was used to immobilize first-row transition metals onto commercially iminodiacetic acid gels. The sodium azide or ethanol preservative was removed by washing the gel three to five times with water. A 75% aqueous slurry was poured into a Bio-Rad 1.0 × 10 cm Econo column or a Pharmacia HR 10/10 column. The resin was washed with 10 column volumes of water. Aliquots of acidified 50 mM metal chloride or metal sulfate solutions were applied to the gel until three-fourths of the column was saturated with metal. In some cases only 100 μmol of metal chloride was added to the column. Cobaltous chloride solutions were prepared anaerobically under a nitrogen atmosphere, and the columns prepared were used immediately with degassed anaerobic buffers to prevent the

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Table I. Metal-Binding Capacities of Commercially Available Chelating Gels (μmol/mL of Gel)

IDA chelating gel	Co(II)	Ni(II)	Cu(II)	Zn(II)
Sephadex G25	39.7	64.1	67.1	48.7
4% agarose	12.0	15.4	16.4	14.3
Sepharose 6B	12.6	15.7	16.4	11.1

formation of Co(III). The metal ion solutions were made appropriately acidic (pH ~5) to prevent the formation of insoluble metal hydroxides. The metal-loaded column was equilibrated with the high-pH buffer (100 mM sodium phosphate, 100 mM NaCl, pH 7.5), then with the low-pH buffer (100 mM sodium phosphate, 100 mM NaCl, pH 4.3), and finally with the high-pH buffer before applying the sample.

This procedure was modified when Zn(II) IMAC columns were prepared. The level of zinc saturation was determined chemically rather than visually since Zn(II) is colorless. Addition of $ZnCl_2$ aliquots to the gel was stopped once free Zn(II) was detected in the eluant fractions. Column fractions were acidified with 1 or 2 drops of 6 N HCl followed by the addition of 4 drops of 0.2 M $K_4[Fe(CN)_6]$ to form the insoluble $Zn_3K_2[Fe(CN)_6]_2$ complex.[12] Large amounts of Zn(II) were eluted during the first wash with the high-pH buffer. No free Zn(II) was detected in any of the other washes or during chromatography with peptide samples. The amount of Zn(II) bound to the gel was estimated from the difference in the amount applied and the amount eluted during the initial water wash and high-pH buffer wash. The latter was estimated by comparing the turbidity of the Zn(II)-containing fractions to the turbidity of standard $ZnCl_2$ solutions after the reaction with potassium ferrocyanide. The sensitivity of this quick detection method is approximately 0.6 mM $ZnCl_2$.

Metal-Binding Capacity. The metal-binding capacity of three commercially available chelating gels was measured in a batchwise manner. Excess metal chloride was added to unsaturated gel (~140 μmol of M^{n+}/mL of gel) in an Eppendorf tube and mixed thoroughly. The metal-loaded gel was washed extensively with water, high-pH buffer, low-pH buffer, and in some cases with water again. Samples washed with water were tested for the presence of Cl^- in the supernatant with $AgNO_3$[12] and then lyophilized to dryness. Aliquots of the wet metal-loaded gels were centrifuged in precalibrated centrifuge tubes to measure the volume of settled gel. The metal content of these wet and dry gels was determined by atomic absorption spectroscopy (Perkin-Elmer 5000) after dissolving the samples in an equal volume of concentrated nitric acid.

IMAC of Peptides. Stock solutions of peptides were prepared at 1 mg/mL concentrations in the pH 7.5 buffer. A 200-μL aliquot was applied to the IMAC column with a peristaltic pump set for a flow rate of ~1 mL/min, which corresponds to a linear flow rate of 76 mL/(cm² h). The column was washed with the same buffer until all unbound material was eluted or until 10 column volumes had passed through the column in cases where the peptide bound. The column was then washed with the pH 4.3 buffer until all bound material was eluted. The effluent was monitored at 210 nm, fractions were collected, and the pH of each fraction was measured. Alternatively, Pharmacia HR 10/10 columns were used with an FPLC LCC500 system. A 200-μL sample was injected on the column followed by washes with buffer A (100 mM sodium phosphate, 100 mM NaCl, pH 7.5) as described above. A pH gradient was generated with the FPLC to elute bound material from the columns by using a 100 mM phosphoric acid, 100 mM NaCl, pH 2.2 mixture for buffer B. The pH of the effluent was measured in a flow-through pH monitor after detection at 214 nm.

Sephadex G25 IDA IMAC columns were used for 10–15 samples without removing the metal ion with EDTA. Cycling from high pH to low pH this often did not affect the reproducibility of peptide chromatographic behavior. The same was not true for the agarose-based resins, as the elution pH varied after cycling through the low-pH gradient two or three times with an eventual loss in ability to bind peptides.

Void and included volumes were measured for Cu(II), Ni(II), and Co(II) Sephadex G25 IDA columns by using high-molecular-weight poly(amino acids), blue dextran, and sulfate. $BaCl_2$ was used to detect sulfate as the $BaSO_4$ precipitate.[12]

Results

Metal-Binding Capacities of Chelating Gels. The metal contents of commercially available chelating gels determined by atomic absorption spectromety on wet swollen samples are shown in Table I. The same general trends in Table I were found for dried samples (not shown), which were subjected to a final water wash to remove salts before lyophilizing the gel samples to dryness. The Sephadex G25 IDA gel had the greatest metal-binding capacity whereas both agarose-based resins had virtually identicial binding capacities for a given metal. The actual metal-binding capacities of the agarose-based resins may be higher than those reported here because of the extensive washing of the gels at low pH, which can result in the acid-catalyzed dissociation of the metal ion from the IDA ligand.[13] This acid-catalyzed dissociation of the metal ion was not observed with the Sephadex G25 IDA resin. Each resin shows the same order of binding capacities for first-row transition metals, i.e. Co(II) < Ni(II) < Cu(II) > Zn(II).

Table II. Elution pH of Histidine-Containing Peptides on M(II) Sephadex G25 IDA Column[a]

peptide	Co(II)	Ni(II)	Cu(II)
His	7.5	7.5	4.7
N-Acetyl-His	7.5	7.5	5.2
Aliphatic			
His-Gly[b]	7.5	5.4	4.5
His-Gly-NH_2	7.5	7.5	4.6
His-Gly-His	5.9	4.8	4.4
His-Ala	7.5	7.5	4.6
His-Val	7.5	5.4	4.4
His-Leu	7.5	5.4	4.5
Gly-His[b]	7.5	5.9	7.5
Gly-His-Gly[b]	7.5	7.5	7.5
Gly-Gly-His[b]	7.5	7.5	7.5
Ala-His	7.5	5.8	7.5
Leu-His	7.5	7.5	7.5
Hydroxy			
His-Ser	7.5	5.4	4.5
Acidic			
His-Glu	7.5	7.5	4.6
Basic			
His-Lys	7.5	5.4	4.6
Aromatic			
His-Phe	7.5	5.2	4.5
His-Tyr	7.5	5.2	4.4
His-Tyr-NH_2	6.4	4.7	4.4
His-Trp	6.4	4.9	4.4
Tyr-His	7.5	5.7	7.5
Sulfur			
His-Met	7.5	5.3	4.6
Met-His	7.5	7.5	7.5

[a] Elution pH is defined as the pH of the individual peptide peak eluting in the pH gradient. The elution pH of these peptides on Fe(II) and Zn(II) Sephadex G25 IDA columns was 7.5 and so are not included in the table. [b] Stability constants for these peptides with Cu(II) and Ni(II) have been measured.

IMAC of Histidine-Containing Peptides. The chromatographic behavior of 21 histidine-containing di- and tripeptides was examined on Fe(III), Co(II), Ni(II), Cu(II), and Zn(II) Sephadex G25 IDA IMAC columns. The elution pH of each histidine-containing peptide reported in Table II is the pH value of the peptide peak that was eluted during a pH gradient from pH 7.5 to pH 4.3. Peptides with an elution pH of 7.5 were eluted within 10 column volumes with characteristic elution volumes (not reported). Peptides with lower elution pHs bound the particular immobilized metal strongly, and an acidic environment was required to displace the bound peptide. The lower the elution pH, the stronger the interaction between the metal ion and the peptide. All 21 histidine-containing peptides were eluted at pH 7.5 from Fe(III) and Zn(II) columns, indicating a lack of strong binding under these conditions. Co(II) bound only three peptides, His-Gly-His, His-Tyr-NH_2, and His-Trp, out of the 21 examined. The

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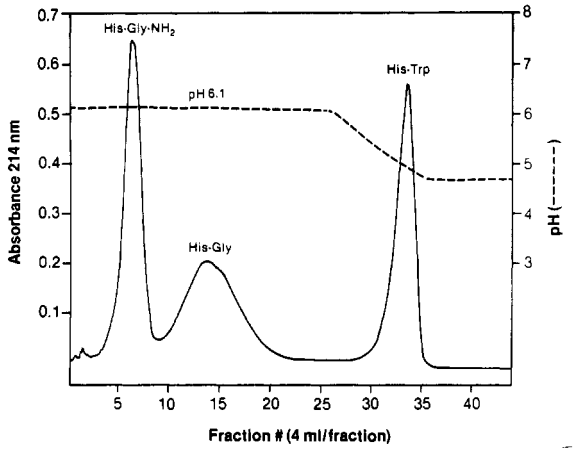

Figure 1. Separation of histidine-containing dipeptides on Ni(II) IDA Sephadex G-25. A 0.2-mL sample containing 80 μg of His-Gly, 75 μg of His-GlyNH_2, and 55 μg of His-Trp was applied to a Ni(II) IDA G-25 Sephadex column equilibrated with 50% B buffer, where the A buffer is 0.1 M Na_2HPO_4, 0.1 M NaCl at pH 7.5 and the B buffer is 0.1 M NaH_2PO_4, 0.1 M NaCl at pH 2.2. A linear gradient from 50 to 65% B over 15 min generated the pH change as shown.

elution pH values on Co(II) were high compared to those determined for Ni(II) and Cu(II). Ni(II) and Cu(II) both bound 13 and 14 out of 21 histidine-containing peptides, respectively, 8 of which were common to both metals.

All 14 peptides with the general formula His-X bound Cu(II) Sephadex G25 IDA and required a pH between 4.6 and 4.4 for elution. The X-His peptides, on the other hand, did not bind under these conditions. Of the nonbinding peptides, Gly-His had the longest retention volume (greater than 1 column volume), followed by Tyr-His. The remaining X-His peptides were eluted within 1 column volume.

The Ni(II) column bound 14 histidine-containing peptides, which were eluted with a range of elution pHs between 5.9 and 4.7. Eleven of the 14 His-X peptides were among this group, the exceptions being His-Gly-NH_2, His-Ala, and His-Glu, which did not bind under these conditions. Three X-His peptides, Gly-His, Ala-His, and Tyr-His, bound immobilized Ni(II) and were eluted with the highest pH of all the peptides bound to Ni(II).

Figure 1 demonstrates the separation of three peptides, His-Trp, His-Gly, and His-Gly-NH_2, on a Ni(II) Sephadex G25 IDA column. The peptides were identified by their previously determined elution positions under the same conditions and HPLC analysis of those peaks.

IMAC of Peptides Containing Lysine and Aspartic Acid. The elution volumes for a series of peptides containing lysine or aspartic acid from Cu(II) and Ni(II) Sephadex G25 IDA columns (Tables III and IV) indicated weaker binding of these peptides to immobilized metals than histidine-containing peptides. The elution volumes for lysine-containing peptides were longer on Cu(II) columns compared to that on Ni(II) columns. Pro-Lys was retained the longest on Cu(II) and Ni(II) columns. In general the peptides containing lysine and an aromatic residue were retained on Cu(II) and Ni(II) columns longer than the other peptides examined, although exceptions can be found easily, the most notable being Gly-Lys for Cu(II). None of the lysine-containing peptides were retained on Co(II) or Zn(II) Sephadex G25 IDA columns.

Aspartic acid containing peptides with the general formula Asp-X were retained the longest on both Cu(II) and Ni(II) columns with Cu(II) binding these peptides with greater affinity than Ni(II), as reflected in the elution volumes. Neither Co(II) or Zn(II) Sephadex G25 IDA columns were able to retard aspartic acid containing peptides.

Discussion

IMAC is simply a series of metal ion ligand substitution reactions, from the first step of preparing the column to the final step of eluting the bound protein or peptide, as shown in Scheme I. The presumed facial orientation of tridentate IDA around Ni(II) is based on solution studies on the stereochemistry of Ni(II) IDA complexes.[14] The solution coordination chemistry of transition metals with amino acids, peptides, and a few proteins has been studied extensively.[15,16] The thermodynamic and kinetic factors governing these interactions have been studied in detail for a few systems.[13] Many detailed mechanisms of metal complex formation have been elucidated by these studies, which points to the inherent difficulty in predicting the extent or rate of complex formation of a given metal ion with an unknown peptide or protein. Stability constants of first-row transition metals with the amino acids have been measured and reveal that amino acids can act

Table III. Elution Volumes $(V_e - V_0)/(V_t - V_0)^a$ of Lysine-Containing Peptides on M(II) Sephadex G25 IDA Columns with 100 μmol of M^{n+}

peptide	Cu(II)	Ni(II)
Aliphatic		
Lys-Gly	1.63	0.74
Lys-Gly-Gly	1.90	1.02
Lys-Ala	1.36	1.16
Lys-Val	2.30	1.45
Lys-Leu	1.08	0.88
	2.17	
Lys-Ile	2.30	1.73
Gly-Lys	3.25	2.56
Ala-Lys	1.09	0.74
	2.44	
Hydroxy		
Lys-Ser	1.77	1.45
Lys-Thr-Tyr	2.17	1.31
Acidic		
Lys-Asp	1.23	1.31
Lys-Glu	1.23	0.88
Lys-Glu-Glu-Ala-Glu	0.28	0.03
Basic		
Lys-Lys	2.17	1.16
Arg-Lys	2.98	2.30
Aromatic		
Lys-Phe	2.71	2.15
Lys-Tyr	2.97	2.44
Lys-Tyr-Ser	1.5	1.59
Lys-Trp	5.13	2.15
Lys-Trp-Lys	4.06	1.87
Imino		
Pro-Lys	5.27	2.58

[a] V_t = 6.4 mL and V_0 = 2.54 mL for Cu(II) column; V_t = 6.68 mL and V_0 = 3.0 mL for Ni(II) column. V_e is the peak elution volume.

Table IV. Elution Volumes $(V_e - V_0)/(V_t - V_0)^a$ of Peptides Containing Aspartic Acid on M(II) Sephadex G25 IDA Columns with 100 μmol of M^{n+}

peptide	Cu(II)	Ni(II)
Aliphatic		
Asp-Gly	3.25	1.16
Asp-Ala	3.38	1.02
Gly-Asp	1.90	0.46
Ala-Asp	0.96	0.46
Val-Asp	0.82	0.46
Aromatic		
Asp-Phe-NH_2	9.26	3.43
Asp-Phe-OMe	9.31	0.74

[a] See footnote *a* of Table III.

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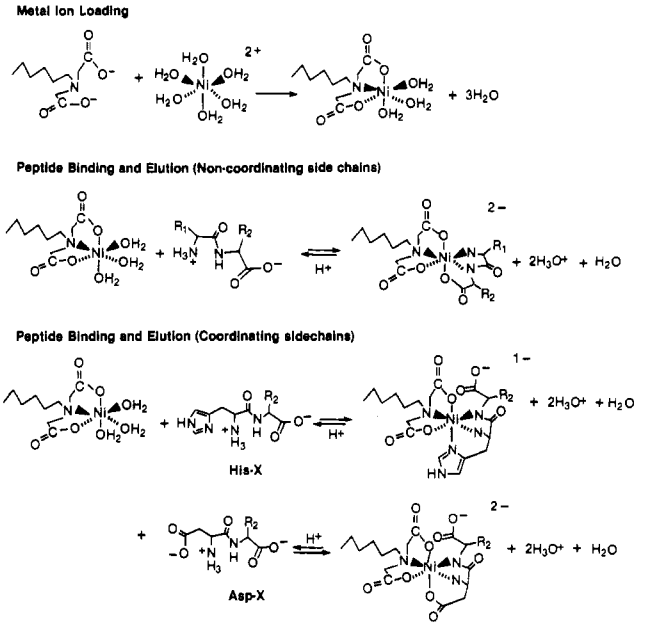
Scheme I



as bidentate ligands through the amino and carboxylate groups, but only a few contain side chains that are capable of tridentate coordination, such as aspartic acid, histidine, and cysteine.[15,17] Structural and potentiometric studies with peptides show that the amide nitrogen itself can be deprotonated in the presence of a metal ion and thereby act as a donor atom.[18,19] The amino acids whose side chains coordinate metals in metalloproteins include those listed above as well as glutamate, glutamine, methionine, tyrosine, and lysine.[20]

The coordination chemistry of immobilized metal ions is a combination of the thermodynamics and kinetics of ternary complex formation between an iminodiacetic acid complex and an incoming ligand, such as a protein, peptide, or amino acid, with the added problem of restricted accessibility of the reactants. The accessibility of large peptides and proteins to immobilized metal ions may be severely restricted by the topographical features of the support resin. In addition to the physical restrictions of the immobilized metal ion, the protein or peptide itself may limit access of the coordinating residues to the metal ion by virtue of its conformation. Third, the metal ion itself may have geometric constraints with respect to the arrangement of the open coordination sites that may not match the orientation of the exposed ligands on the polypeptide.

The kinetics of these ligand substitution reactions must be fast in order to observe binding under these chromatographic conditions. If a peptide or protein has a very large affinity for an immobilized metal ion but a slow rate of complex formation then it will appear as if the protein has no affinity for the metal ion under these rather stringent conditions. Preliminary experiments with high-affinity peptides chromatographed at different flow rates indicated that the rate of complex formation was not a limiting factor. The rates of ligand exchange for first-row transition metals are generally quite fast, so the kinetics are not expected to dominate the observed chemistry although steric effects can substantially inhibit a normally facile reaction.[13]

Our goal in studying the chromatographic behavior of peptides was to find or design an amino acid sequence with a high affinity for immobilized metal ions, attach that chelating peptide (CP) to the N terminus of a polypeptide, and purify that CP-polypeptide using IMAC. The first step in designing such a chelating-peptide sequence was to determine the types of amino acids that form strong coordinate-covalent bonds with transition metals under chromatographic conditions and to gauge the steric restrictions imposed by such a system. The documented chemistry of transition metals with amino acids, peptides, and proteins can be used as a guide to choose amino acids for investigation as potential ligands.[17] Porath's work on IMAC of amino acids and peptides could also be used to choose the amino acid composition of potential chelating peptides.[9] A comparison of the chromatographic behavior of peptides within the same class (i.e. histidine-containing peptides) should reveal any steric hindrance these coordinating residues encounter on binding immobilized metal ions and the inherent specificity and selectivity of a peptide.

Immobilization of the metal ion decreases the number of available coordination sites from 6 to 3 and therefore the required length of a chelating peptide. The ϵ-amino group of lysine, the β-carboxyl group of aspartic acid, and the histidyl imidazole ring function as Lewis bases at pHs above their pK_a and can bind transition metals which function as Lewis acids. The peptide backbone also contains potential donor atoms for coordinate-covalent bonds such as the α-amino group, C-terminal carboxylic acid, and the amide or peptide bond itself. Thus simple dipeptides contain a sufficient number of donor atoms to fill all the open coordination sites on an IDA immobilized metal ion (Scheme I).

IMAC of Peptides. Peptides containing lysine and aspartic acid interacted weakly with Ni(II) and Cu(II), as reflected by the large elution volumes, but did not bind tightly enough to require a low-pH environment for elution. Peptides with an N-terminal aspartic acid residue are capable of forming a six-membered ring with the metal ion, α-amino group, and β-carboxyl group, thereby stabilizing the complex,[15,19] to which we attribute the even longer retention times observed for these peptides (Scheme I). Peptides containing an aromatic residue also exhibited long retention times. These results indicate that Cu(II) IMAC could be used for purifying peptides with N-terminal residues capable of forming six-membered rings, from other peptides lacking this structural feature. If a protein has an N-terminal aspartic acid residue, this same interaction may make a small contribution to the adsorption of that protein to an IMAC column. It is unlikely that aspartic acid or lysine residues are the major determinants of protein binding because of the poor binding characteristics of these residues in small peptides, where steric restrictions are minimal.

Peptides with the formula His-X bound Cu(II) very tightly whereas X-His peptides did not bind since they were eluted at pH 7.5. The low and narrow elution pH range of His-X peptides were indicative of tight peptide binding to immobilized Cu(II) and a common mode of binding. Cu(II) forms square-planar or distorted octahedral complexes, so only two open coordination sites are available once Cu(II) has been immobilized. Formation of a chelate ring requires two coordination sites, and His-X peptides can form a six-membered ring, with the metal ion, N-terminal amine, and imidazole δ-nitrogen. This structural feature seems to confer stability to the Cu(II) complexes formed, which made their dissociation difficult and required high proton concentrations. It should be noted that the elution pH values of 4.4–4.6 are much lower than the pK_a of ~6 for histidyl imidazole groups. The apparent pK_a of the imidazole group seems to be lowered and depends on the strength of the metal–ligand interaction. Six-membered chelate ring coordination is common to all metal peptide complexes of histidine whose crystal structures have been determined, except Cu(II), where the imidazole nitrogen does not participate in coordination at low pH.[21] The crystal structure of the $(Gly\text{-}His)_2$ Cu(II) complex shows a six-membered ring is formed between the amide nitrogen and the imidazole ring.[22] Gly-His does not bind immobilized Cu(II) IDA under the chromatographic conditions used in this investigation. This is probably due to the decreased thermodynamic stability of chelate rings

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formed with amide nitrogen compared with N-terminal amines.[23] This is also supported by the higher elution pH of *N*-acetylhistidine compared to that of histidine on Cu(II).

Other peptides whose chromatographic behavior is reported here and whose stability constants have been measured are His-Gly, Gly-Gly-His, and Gly-His-Gly.[17] These stability constants do not necessary correlate with the degree of binding to immobilized metal ions as the complexes formed in solution and on the resin are not the same. Gly-His-Gly forms a bis complex with Cu(II) in solution with very high affinity (log K = 19.95) but fails to bind immobilized Cu(II) IDA, which is incapable of forming an immobilized bis complex. The same peptide can also form a trinuclear Cu(II) complex with an even greater stability constant, which the resin prevents from forming.

Ni(II) is also capable of forming six-membered rings with N-terminal histidyl peptides,[19] and this mode of coordination is probably responsible for the low elution pH values measured for many of the His-X peptides studied. However, there were exceptions such as His-Glu and His-Ala, which did not bind immobilized Ni(II). The wide range of elution pH values reflects the varying degree of affinities of histidine-containing peptides for immobilized Ni(II), which is probably related to the number and arrangement of open coordination sites. Ni(II) forms six-coordinate octahedral complexes whereas Cu(II) prefers a four-coordinate square-planar geometry. After immobilization through iminodiacetic acid, Ni(II) still has three open coordination sites and Cu(II) has only two. Two coordination sites are occupied by the chelate ring with the N-terminal amine and imidazole nitrogen, and the third position is probably occupied by the amide nitrogen in peptides lacking a second coordinating side chain. The peptide conformation required for tridentate coordination around Ni(II) may lead to steric repulsions depending on the nature of the side chain of the neighboring amino acid, thereby giving rise to a range of peptide elution pH values on Ni(II). We are in the process of evaluating the contribution of steric factors in determining affinities of the ternary complexes form, by using molecular mechanics.

Gly-His and Ala-His bound Ni(II) weakly, which may involve deprotonation of the peptide bond to form a six-membered chelate structure with the imidazole ring. Glycine and alanine are relatively small so that the amide may be more accessible for binding compared to bulkier residues. The presence of the amide group in His-Gly-NH_2 seemed to destroy binding of His-Gly to Ni(II) but enhanced it in the His-Tyr case. The affinity of histidine-containing peptides for Ni(II) is more sensitive to the identity of nearby residues and seems to be well-suited for separating similar peptides with slightly different affinities, compared to the case for Cu(II), whre the peptides can be divided into two classes, those that bind and those that do not bind Cu(II).

Peptides containing both histidine and an aromatic residue bound the immobilized metal ions stronger than the other peptides. In fact, the separation of His-Trp from other peptides was effected on the basis of its high affinity for Ni(II) as shown in Figure 1. The unusually high affinities of His-Trp, His-Tyr-NH_2, and His-Gly-His, compared to those of the other peptides, may be due to a stacked conformation of the aromatic rings in these peptides.[24] Stacking interactions are known to enhance the rate of Ni(II) complex formation in a series of ternary complexes with terpyridine and other aromatic nitrogen-donating ligands.[13] In addition to these systems there are many biological systems in which stacking interactions may play an important role. A pertinent example is the Cu(II) ternary complexes with aromatic amines and aromatic amino acids reported recently, which are stabilized by stacking interactions and have important implications for biological recognition and function.[25,26] In addition to van der Waals interactions between the aromatic rings, charge-transfer interactions involving the aromatic rings, histidyl imidazole, and metal ion orbitals could lead to added stabilization of the peptide metal complex.

Clearly there are many factors controlling the interaction of a peptide with an immobilized metal ion. The presence of a histidine residue is not sufficient for peptide binding to immobilized metal ions because the position of the histidine in the peptide profoundly influences it binding behavior. Some general trends that were found by studying the peptide chromatographic behavior are that peptides with the formula His-X will bind Cu(II) strongly and most will bind Ni(II) to a lesser extent. A few peptides with X-His structure also bound Ni(II) but rather weakly. Of the 21 peptides examined, three had unusually high affinities for immobilized metal ions. These were His-Gly-His, His-Tyr-NH_2, and His-Trp.

Studying the chromatographic behavior of peptides containing residues with potential donor atoms led to the identification of chelating-peptide sequences with high affinities for immobilized transition metals. The peptide chromatography hinted at the selectivity and specificity of IMAC that can be achieved with simple peptides. The second step in proving the feasibility of this hypothesis is to purify a polypeptide or protein containing an N-terminal CP, such as His-Gly-His, His-TyrNH_2, or His-Trp, which has been accomplished[11] and will be published in detail. The third step of evaluating the utility of CP-IMAC is the purification of a chimeric CP-protein synthesized in an appropriate host organism, which has also been carried out[27] and will be published in detail. CP-IMAC is expected to be most useful for biosynthetic proteins made by recombinant DNA techniques since the additional nucleotide sequence coding for the CP can be cloned in tandem with the desired protein to form a chimeric protein suitable for CP-IMAC. This method has the potential to eliminate the need for separate purification schemes for each protein synthesized by recombinent DNA technology.

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