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DIFFERENTIAL INTERACTION OF PEPTIDES AND PROTEIN SURFACE STRUCTURES WITH FREE METAL IONS AND SURFACE-IMMOBILIZED METAL IONS

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SUMMARY

We have examined the influence of free metal ions on the affinity of structurally-defined proteins and peptides for model surface-immobilized metal ions. The model proteins chosen differed widely in both the type and quantity of surface-accessible electron donor groups. Metal ion affinity chromatography and equilibrium binding analyses demonstrated that the presence of excess free Cu(II) ions did not measurably affect either the affinity or the binding capacity of lysozyme for immobilized iminodiacetate–Cu(II). Similarly, the presence of excess free Cu(II) ions did not detectably affect the chromatographic behavior or measured affinity of either copper-saturated lactoferrin or iron-saturated lactoferrin for the immobilized Cu(II) ions. Its binding capacity however, was diminished. The affinities of small peptides for immobilized Cu(II) ions was found to be related to their number of His residues. Peptides with 0, 1, 2 and 3 His residues were resolved by high-performance immobilized Cu(II) affinity chromatography in both the presence and absence of added Cu(II) ions. In the presence of excess free Cu(II) ions, however, retention (affinities) of these peptides by immobilized Cu(II) ions was increased in relation to their number of His residues. These data demonstrate that protein surface binding sites for free and immobilized metal ions are functionally distinct. The presence of free and/or protein surface-bound metal ions does not preclude interaction with the same immobilized metal ions. Stationary phase immobilized metal ions can be a useful model system through which we can better understand the influence of macromolecular surface-immobilized metal ions on macromolecular recognition events. The significance of these findings are also important to the design of other site-specific and domain-specific affinity reagents involving metal ions.

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INTRODUCTION

Metal ions *in vivo* are largely associated with protein and other macromolecular surfaces[1]. Several of these protein–metal ion interactions, especially those of high affinity, are well-characterized. Yet, little is known of the manner in which the interaction of metal ions with specific regions of a protein surface may directly influence that protein's association with other proteins and neighboring macromolecules. Specifically, biologically important protein–protein interactions, facilitated or stabilized by surface-immobilized metal ions, may be viewed and evaluated as potential regulatory events. Metal ions that are partially chelated and immobilized on a macromolecular surface (*e.g.*, protein surface) may alter or fine tune subsequent macromolecular recognition events. The extent to which available free metal ions effectively compete with, or interrupt, these processes is not known. Similar considerations are most important for the interpretation of peptide and protein retention data obtained by immobilized metal ion affinity chromatography.

Well-defined model systems are needed for analytical investigations of protein interactions with surface-immobilized metal ions. Although stationary phase immobilized metal ions have existed for some time, they have been used almost exclusively for chemical separation and purification. Separations of amino acids and peptides[2–4] then preceded the group fractionation of proteins[3–5]. Contributions by Porath and co-workers[5,6] greatly facilitated the widespread use of immobilized metal ions for the affinity purification of proteins. In contrast to their use for protein purification, we have used metal chelating stationary phases of the type first described by Porath as analytical probes to evaluate protein surface structure/function relationships[7–14]. In an effort to quantify these observations, equilibrium binding experiments were performed to measure alterations in immobilized metal ion–protein binding capacities and apparent thermodynamic association constants as a function of protein surface structure and function [8,10]. Ambiguities often associated with unknown degrees of trace metal ion contamination and metal ion transfer events during sample preparation and isolation, as well as during immobilized metal ion affinity chromatography, have raised important questions about the effects of free metal ions on our ability to measure and characterize immobilized metal ion binding sites on a protein surface. The influence of free or competing metal ions on the equilibrium interactions between proteins (and peptides) and immobilized metal ions, however, has *not* been evaluated *per se*. At least one report[15] actually specified the addition of free metal ions to the buffer systems used during immobilized metal ion affinity chromatography to compensate for metal ion leakage and to facilitate the subsequent column renegeration process. However, in other presentations, the competing effects of metal ions, present as a result of either metal ion "scavenging" by the protein or simple metal ion leakage from the stationary phase, are assumed to be negative[16]. These effects remain hypothetical.

We have developed several model systems to evaluate the influence of soluble (free) Cu(II) ions on the affinity of specific proteins and peptides for other surface-bound (*e.g.*, agarose bead surface-immobilized or protein surface-immobilized) transition metal ions. Lysozyme is a good model protein for these investigations because its structure is known, its chromatographic properties on a wide variety of other stationary phases are well-characterized[17] and it has been shown by Scatchard analy-

ses of equilibrium binding data to interact with immobilized Cu(II) ions via a single class of intermediate affinity interaction sites[8]. Lactoferrin was chosen as a more complicated model protein for these investigations. It is a glycoprotein of much larger mass (78 000 daltons) with several surface-accessible electron donor groups including His, Trp, Phe and Tyr residues. The crystallographic structure of human lactoferrin has recently been refined to 2.8 Å; therefore, its metal-binding properties and biological activities can now be evaluated from a structural perspective[18–20]. It is the most abundant protein of human colostral whey[21]. In addition to its well-known iron-binding properties, however, lactoferrin in human milk (80 to 90% iron-free) is believed to play an important role in the bioavailability of copper and zinc ions[22]. This propensity may also affect the other known biological properties of lactoferrin: its ability to promote cell growth[23,24], modulate immune cell interactions[25], inhibit bacterial growth[26] or bind DNA[27,28]. We are interested in using immobilized metal ions as a probe to evaluate structural changes expressed on the surface of lactoferrin as a result of its interaction with DNA, iron and other transition metal ions, particularly copper.

Finally, it is important to know if free metal ions affect the interaction of small peptides with immobilized metal ions. We have developed high-performance immobilized metal ion affinity chromatography into a method for the capture and separation of peptides and proteolytic digestion products under conditions compatible with preserved biological activity[12,14,29]. The retention of peptides (up to approximately 5000 daltons) on columns of immobilized Cu(II) ions has been shown to be predicted by the number of His residues[14]. Therefore, we chose several small peptides containing various numbers of His residues (0–3) as model peptides for these investigations.

MATERIALS AND METHODS

Lactoferrin was purified to homogeneity from human colostrum using immobilized single-stranded DNA as a one-step affinity purification method[28]. Metal ions were removed from lactoferrin by incubation/dialysis against low pH buffers[22] (1 *M* sodium phosphate at pH 4.0–4.2 with 1 mg/ml ascorbic acid) using desferrioxamine mesylate (Sigma, St. Louis, MO, U.S.A.) as the iron chelator[30]. To produce iron-saturated lactoferrin, sodium bicarbonate was added to a concentration of 0.1 *M*, then ferrous sulfate in 0.01 *M* hydrochloric acid was added before incubation at 37°C for 15 min or longer. ^{59}Fe (DuPont, NEN) was sometimes added as a radiolabeled tracer to monitor iron dissociation and/or hololactoferrin elution properties. To make copper-saturated lactoferrin, copper(II) sulfate in water was used instead of ferrous sulfate. Excess metals were removed by dialysis. Saturation was monitored by spectral analysis at 465/280 nm for the iron-saturated form and at 430/280 nm for the copper-saturated (iron-free) form. The protein-bound metal ion contents were confirmed by energy-dispersive X-ray fluorescence spectroscopy (William E. Seifert, Analytical Chemistry Center, University of Texas Health Science Center, Houston, TX, U.S.A.). Lysozyme from chicken egg white was obtained from Sigma. The synthetic peptides, oxytocin, angiotensin I and II, and parathyroid hormone (1–34) were obtained from Peptide Institute, Osaka, Japan (the kind gifts of Drs. Nakagawa and Sakakibara).

All experiments were performed at room temperature. Chelating Sepharose

Fast Flow (Pharmacia, Sweden) was packed into a 2.55 × 1 cm I.D. (bed volume, 2 ml) column. After the metal chelating stationary phase [iminodiacetate (IDA)–agarose] was washed with water, 50 m*M* copper sulfate in water was passed through the column. Excess metal was washed away with 0.1 *M* sodium acetate and 0.5 *M* sodium chloride at pH 3.5. A flow-rate of 25 ml/h was used. For the evaluation of lysozyme interaction with immobilized Cu(II), two different column equilibration and elution buffer systems were used. The immobilized Cu(II) columns were equilibrated with either 20 m*M* sodium phosphate (pH 7.0) containing 0.5 *M* sodium chloride, or 100 m*M* sodium acetate (pH 6.0) containing 0.5 *M* sodium chloride. The column equilibration buffers also contained 10 μM copper sulfate as noted. The sample was loaded onto the column, which was then developed with a linear gradient of either (1) 0.1 *M* sodium phosphate, 0.5 *M* sodium chloride (± 10 μM copper sulfate) from pH 7.0 to 3.6, or (2) 0.1 *M* sodium acetate, 0.5 *M* sodium chloride (± 10 μM copper sulfate) from pH 6.0 to 3.5. For the lactoferrin studies, the sodium acetate column equilibration and elution buffers were used. Note that these buffers were modified to include 3 *M* urea and 100 μM copper sulfate as indicated. The total gradient volumes were 30 ml and 1-ml fractions were collected. The absorbance at 280 nm and the pH were measured continuously.

High-performance immobilized metal ion affinity chromatography of the model peptides was performed as previously described[29]. Replicate evaluations of apolactoferrin by high-performance metal ion affinity chromatography were performed exactly as described earlier[28] with mobile phase buffers containing 3 *M* urea. Ultrapure urea was obtained from Bethesda and used after being passed through a mixed function deionizing resin (AG 501-X8 (D) from Bio-Rad). The TSK chelate 5PW (10 μm, 750 × 7.5 mm, I.D.) columns, prepared with iminodiacetate functional groups[31], were a gift from Dr. Kato of Tosoh, Japan. The Beckman System Gold high-performance liquid chromatograph (Fullerton, CA, U.S.A.) was used with a Beckman Model 166 or 167 flow-through ultraviolet spectrophotometer. A Sensorex (Stanton, U.S.A.) flow cell and a Corning (U.S.A.) pH meter were used to monitor the column effluent pH continuously.

Equilibrium binding studies and Scatchard analyses of data were performed as described previously[8]. All assays were performed at room temperature with 20 m*M* sodium phosphate, 0.5 *M* sodium chloride, pH 7.0 in the absence and presence of free copper ions.

Lysozyme and lactoferrin protein structures were evaluated using a modified version of the original program FRODO[32] (version 6.6) and the program ACCESS[33] (version 2 by B. Lee, F. M. Richards, T. J. Richmond, and M. D. Handschumacher, Yale University, New Haven, CT, U.S.A.) on a VAX 8810 computer equipped with an Evans and Sutherland PS390 molecular graphics terminal. PS 300 FRODO version 6.6 was written by James W. Pflugrath, John S. Sack, and Mark A. Saper in the laboratory of Florante A. Quiocho at the Department of Biochemistry, Rice University, Houston, Texas. Lysozyme coordinates (LYZ1PDB) were obtained from the Broohaven Protein Data Bank. The refined coordinates for human lactoferrin[19] were generously provided by Dr. Edward N. Baker at Massey University, New Zealand.

RESULTS

Proteins are known to interact with transition metal ions via a select group of solvent-accesible amino acid residues[1]. For each of the model proteins selected for these investigations, we have calculated the surface-accessible areas[33] of those amino acid residues proposed to interact with both free and immobilized transition metal ions[1,5]. A spherical probe with a radius equal to that of a water molecule (1.40 Å) was used to calculate the contact surface area in square Ångströms. The contact areas of each atom of a given residue were summed. Lysozyme contained 1 His, 1 Phe, 3 Trp and 3 Tyr residues with contact surface areas above 25 $Å^2$. Lactoferrin contained 4 His, 9 Phe, 3 Trp and 4 Tyr residues with surface areas at or above 25 $Å^2$. There are no free sulfhydryl groups on the surface of either protein.

The effects of added free Cu(II) ions on the ability of lactoferrin to interact with immobilized Cu(II) were first monitored by immobilized metal ion affinity chromatography. To ascertain whether metal ion ligands in the two high affinity iron-binding sites of lactoferrin were participating in the recognition of the immobilized Cu(II) ions, prior to chromatography, these binding sites (in the N-lobe and C-lobe) were loaded with copper or iron. Dialysis was performed to remove free and loosely bound metal ions. Equilibrium sedimentation analyses performed before and after exposure to metal ions confirmed the monomeric status of lactoferrin in buffers used for these investigations. Immobilized Cu(II) affinity chromatography of the copper-saturated lactoferrin and iron-saturated lactoferrin was performed in the presence and absence of 100 μM copper sulfate in the column equilibration and elution buffers. The copper-saturated (Fig. 1A) and iron-saturated (Fig. 1B) forms of human lactoferrin were tightly bound to the stationary phase IDA–Cu(II) ions in both the absence and presence of added free Cu(II) ions. Indeed, no decrease in lactoferrin affinity or apparent capacity for the immobilized Cu(II) ions was detected in the presence of the added Cu(II) ions (Fig. 1A and 1B). The influence of excess free Cu(II) ions on the affinity of iron-saturated lactoferrin for immobilized Cu(II) ions was also investigated in the presence of 3 M urea as a mobile phase modifying reagent. The presence of 3 M urea decreases the affinity of lactoferrin for the immobilized Cu(II) ions (Fig. 1C). Under these conditions, however, lactoferrin affinity for the immobilized Cu(II) ions was still unaffected by the presence of 100 μM copper sulfate in both the column equilibration and elution buffers. The lactoferrin-bound ^{59}Fe was quantitatively recovered and constant specific activity was maintained. There was no exchange of Cu(II) ions with the protein-bound ^{59}Fe.

We next attempted to document the extent to which Cu(II) ions were transferred from the stationary phase IDA chelating groups to lactoferrin during the metal ion affinity chromatography. Lactoferrin, free of any bound metal ions (*i.e.*, apolactoferrin), was subjected to repetitive chromatography on a high-performance immobilized Cu(II) affinity column. After each of four successive passages through the immobilized Cu(II) ion column (Fig. 2A), the apolactoferrin was evaluated for bound Cu(II) ions by ultraviolet and visible spectral analysis (Fig 2B) and energy-dispersive X-ray fluorescence spectroscopy (data not shown). No lactoferrin-bound Cu(II) ions were detected by either analytical method.

Even though the chromatographic results suggested that metal ion binding sites on the surface of lactoferrin for free Cu(II) ions were not those contributing to the

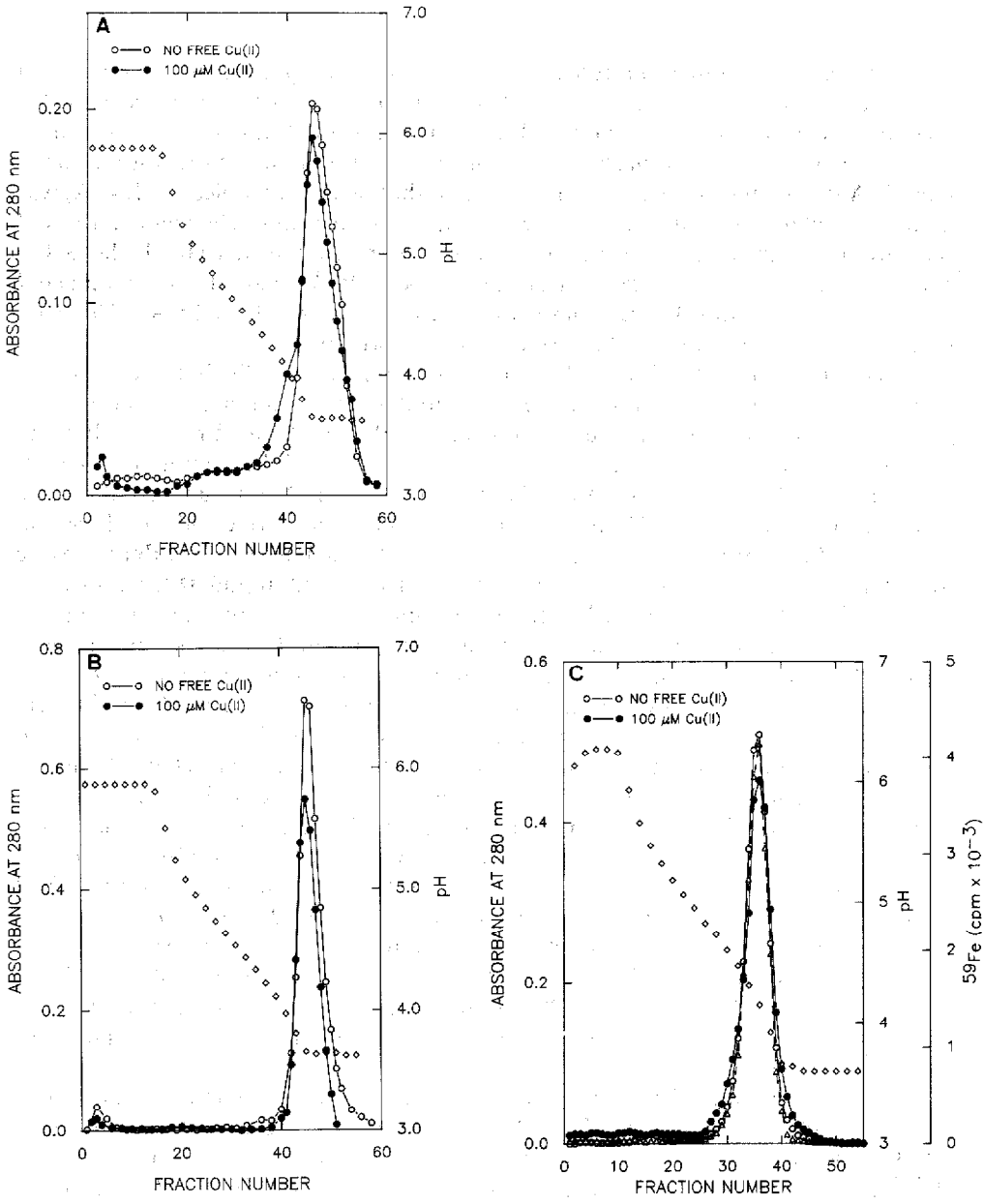


Fig. 1. Effect of free Cu(II) ions (100 μM copper sulfate) in the column equilibration and elution buffers on the chromatographic behavior of copper-saturated lactoferrin (A), and iron-saturated lactoferrin (B and C) on affinity columns of IDA–Cu(II). The profiles shown in A and B were generated using acetate buffer systems without added urea. The profiles shown in C were generated using the same buffer systems except that 3 M urea was included. The pH (◇) and ^{59}Fe radioactivity (△) were measured in each of the collected fractions. All of the ^{59}Fe radioactivity eluted was lactoferrin-bound and recovery was quantitative in both the presence (102%) and absence (106%) of 100 μM copper sulfate. Details are provided in Materials and methods.

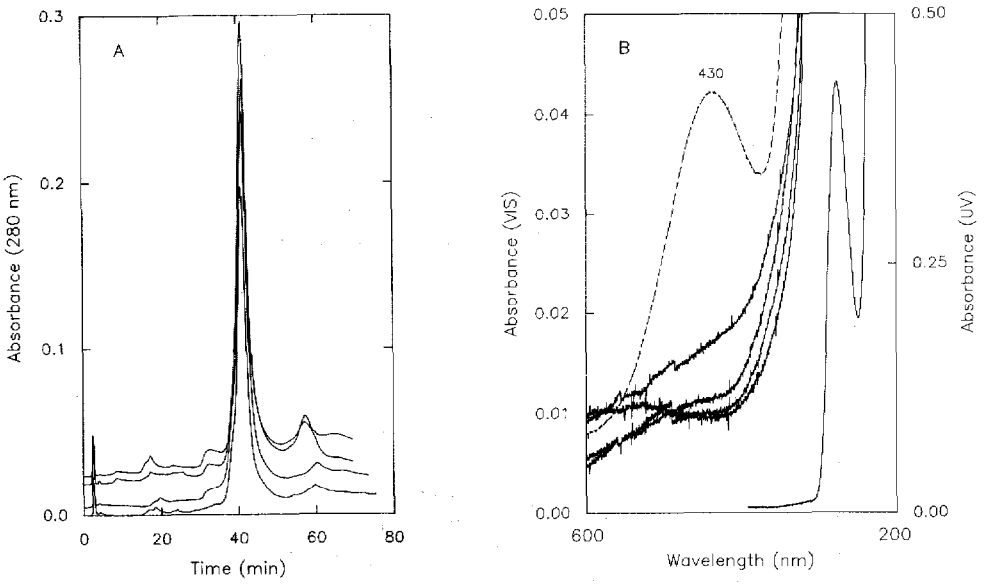


Fig. 2. (A) Repetitive isolation and rechromatography of apolactoferrin using a high-performance IDA–Cu(II) affinity column. (B) UV–VIS absorption profile of the isolated apolactoferrin peaks shown in A. The dotted line represents the visible absorption spectrum of Cu(II)-saturated lactoferrin. The metal ion composition of the apolactoferrin peaks shown was determined by energy dispersion X-ray fluorescence.

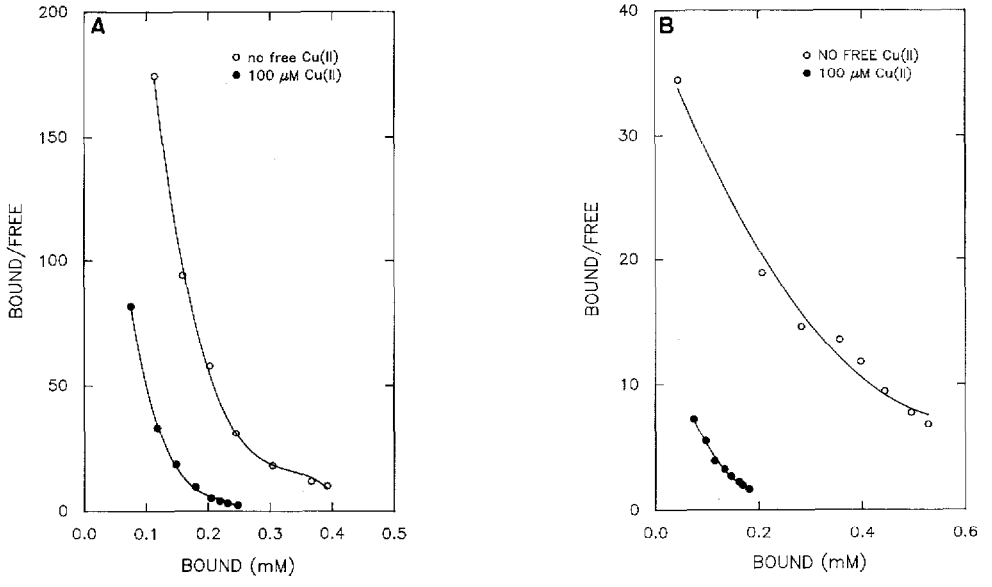


Fig. 3. Scatchard analysis of the equilibrium interaction between (A) copper-saturated lactoferrin or (B) iron-saturated lactoferrin and IDA–Cu(II) affinity gel particles in the absence and presence of 100 μM copper sulfate.

interaction with immobilized metal ions, a quantitative evaluation was undertaken. The chromatographic evidence for the presence of distinct protein surface binding sites for free *versus* immobilized metal ions was supported by Scatchard analyses of equilibrium binding data. The Scatchard plots in Fig. 3 show that the affinities of copper-saturated lactoferrin (Fig. 3A) and iron-saturated lactoferrin (Fig. 3B) for Cu(II) ions immobilized on the IDA-agarose beads were the same in the presence and absence of excess Cu(II) ions (*i.e.*, 100 μM copper sulfate). The curvilinear Scatchard plots observed for lactoferrin likely reflected the participation of more than one class of immobilized Cu(II) ion binding sites. Extensive participation may be anticipated from the large number and type of surface-accessible electron donor groups distributed over the surface of lactoferrin.

We next evaluated the simpler model protein, lysozyme. The chromatographic behavior of lysozyme on columns of immobilized Cu(II) ions was entirely unaffected by the simultaneous presence of 10 μM copper sulfate (Fig. 4). This observation appeared independent of buffer ion effects on lysozyme affinity for the immobilized Cu(II) ions. Regardless of whether the lysozyme was eluted within the pH gradient (acetate buffer system) or at low pH values near the end of the pH gradient (phosphate buffer system), the presence of free Cu(II) ions had no appreciable effect. Similarly, the equilibrium dissociation constant (k_d = 33 μM) and binding capacity (Fig. 5) of lysozyme for the immobilized Cu(II) ions, like those of lactoferrin, were not affected by the presence of excess (10 μM) free Cu(II) ions.

Finally, the influence of free Cu(II) ions on the interaction of model peptides with immobilized Cu(II) ions was evaluated by high-performance immobilized metal

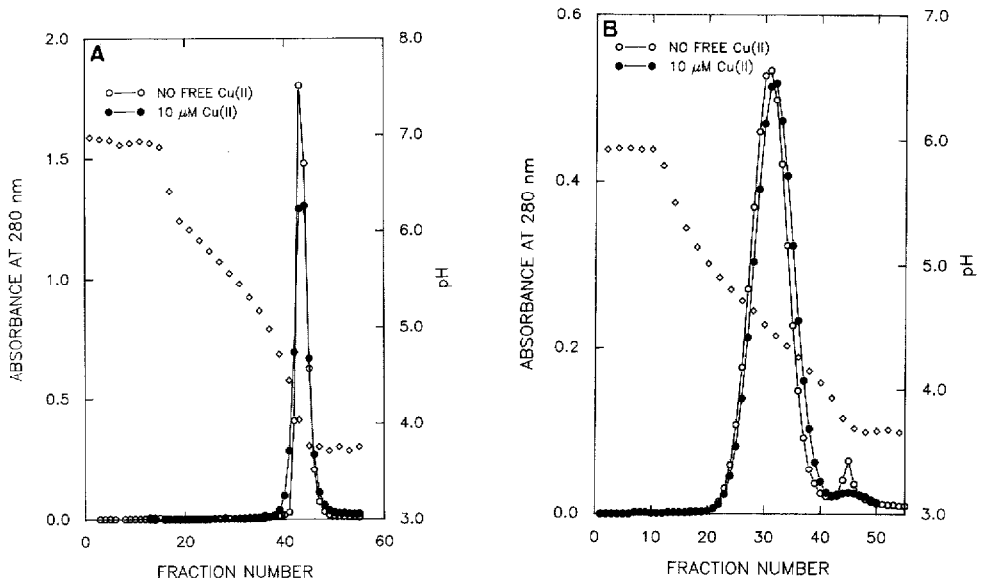
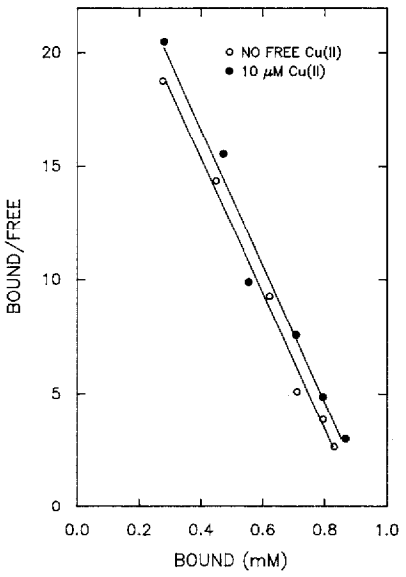


Fig. 4. Effect of free Cu(II) ions (10 μM copper sulfate) in the column equilibration and elution buffers on the chromatographic behavior of lysozyme on affinity columns of IDA–Cu(II). A illustrates the elution properties observed using the phosphate buffer system and B shows the elution profiles obtained using the acetate buffer system. ◇ = pH. Details are provided in Materials and methods.



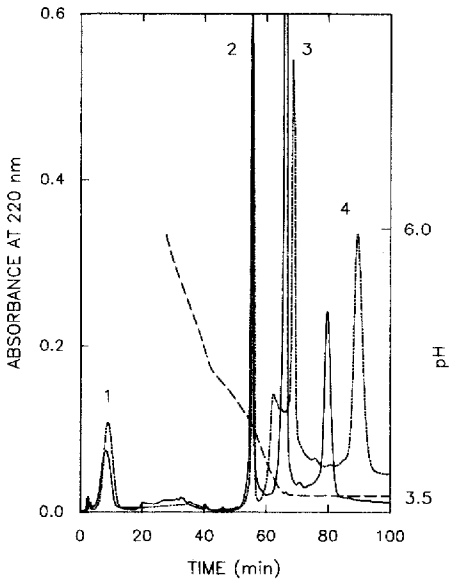


Fig. 5. Scatchard analysis of the equilibrium interaction between lysozyme and IDA–Cu(II) affinity gel particles in the absence and presence of 10 μM copper sulfate.

Fig. 6. High-performance immobilized Cu(II) affinity chromatography of oxytocin (1), angiotensin II (2), angiotensin I (3) and parathyroid hormone (1–34) (4) in the presence and absence of 10 μM Cu(II) ions in the column equilibration and elution buffers. — = No free metal; - - - - = 10 μM Cu(II); - - - = pH.

ion affinity chromatography. Oxytocin, angiotensins I and II, and parathyroid hormone (1–34) were chosen from among the small peptides to represent peptide classes with increasing numbers of histidine residues in their sequences. A high-performance immobilized Cu(II) column separated them essentially according to the number of histidine residues in the peptide structures (Fig. 6). This phenomenon has been studied more thoroughly with a much larger sample of peptides[14]. The addition of free metal ions to the peptide samples (50 μM copper sulfate) and column buffers (10 μM copper sulfate) actually appeared to increase the retention of peptides on the immobilized Cu(II) column. This increased retention was most apparent for peptides with higher numbers of histidine residues (Fig. 6).

DISCUSSION

It is important to understand both the structural and immediate environmental factors that determine the specificity and affinity with which trace metal ions interact with protein surfaces. Furthermore, to understand metal ion bioavailability in biological systems and metal ion-dependent alterations in protein quaternary structure and function, we must characterize the influence of soluble (free) metal ions on the equilibrium interaction of peptides and proteins with other macromolecular surface-immobilized metal ions. These processes are not currently understood. Although the interaction of proteins with transition metal ions in solution has been investigated for many years[1], analytical investigations of protein interactions with stationary phase immobilized metal ions are quite limited.

In their first paper on the subject of immobilized metal ion chromatography for protein separations, Porath *et al.*[5] speculated on the amino acid residues responsible for protein interactions with transition metal ions[1]. These discussions have been reviewed by Sulkowski[34]. More recently Hemdan *et al.*[16] have confirmed these expectations in the case of His residues. It has been suggested, therefore, that immobilized transition metal ions may be viewed as a probe for protein surface-accessible His residues (*i.e.*, in the absence of any other surface-accessible electron donor groups). Most intracellular proteins are not as simple as the proteins chosen by Hemdan *et al.* and, even in the case of extracellular proteins with solvent-exposed His residues, we must avoid oversimplifications based upon chromatographic elution data alone.

Several factors must influence the relative contributions of potential metal chelating residues (electron donor groups) on the surface of a protein (*e.g.*, Cys, His, Trp, Phe, Tyr) toward the affinity of that protein for immobilized metal ions. The obvious has been stated. Much depends upon the number and surface accessibility of the immobilized metal ion interaction sites. Less obvious and an often neglected consideration is one of protein geometry. In the absence of protein denaturation or other similar drastic alterations in secondary and tertiary structure at the immobilized metal ion stationary phase interface, geometrical constraints appear to demand that not all surface-exposed interaction sites may participate in the protein immobilization event. Furthermore, because of variations in the local environment for any given type of surface interaction site (*e.g.*, pK_a of His groups) caused by near-neighbor relationships, not all 1-His or 2-His type interactions may be of equal or even similar affinity. For example, even though the protein ligands and counterions participating in the N-lobe and C-lobe iron-binding clefts of lactoferrin are identical, the affinities of these two sites for iron are different[19,20]. Thus, even those proteins with only a single type and number of surface-accessible interaction sites, *e.g.*, His residues (*i.e.*, no surface accessible Cys, Trp, Tyr or Phe residues), would not be expected to have a similar affinity for immobilized metal ions. These considerations are further complicated by the unknown contribution of competing free metal ions. It has been stated that free metal ions that are "scavenged" from the IDA–agarose column during chromatography may occupy the protein's surface-accessible metal ion binding site thus rendering it useless for protein immobilization[16]. Thus, the present study was a first attempt to document and quantify further the influence of free metal ions on the interactions of peptides and proteins with surface-immobilized metal ions. A distinct feature of these investigations was the use of sample peptides and proteins with several levels of known structural complexity.

The presence of excess free metal ions in samples and buffers for column chromatography and equilibrium binding studies appeared not to compromise protein affinity for the same immobilized metal ions. These findings were consistent regardless of gradient elution position and were confirmed under a variety of experimental conditions (*i.e.*, acetate, phosphate, ± urea). Scatchard analyses of our equilibrium binding data showed that the apparent lack of effect of free metal ions is most evident with a relatively simple model protein such as lysozyme, which has a single class of sites operational during its interaction with the immobilized Cu(II) ions. However, both copper-saturated lactoferrin and iron-saturated lactoferrin were tightly bound to immobilized Cu(II) even in the presence of excess Cu(II) ions. In the case of ^{59}Fe-labeled lactoferrin, no ^{59}Fe was displaced during the immobilized Cu(II) ion

affinity chromatography. The specific metal ligand binding sites in the N-lobe and C-lobe iron-binding clefts of lactoferrin, therefore, do not contribute to the interaction of lactoferrin with immobilized Cu(II) ions. Furthermore, apolactoferrin was bound to the immobilized Cu(II) and eluted with no detectable bound copper. Although clearly demonstrated in the case of carboxypeptidase[35], the protein-mediated "stripping" or "scavenging" of immobilized metal ions [at least for Cu(II)] during immobilized metal ion affinity chromatography may not be as common as is frequently suggested.

We do not presently understand why the presence of excess free metal ions actually seemed to promote the interaction of some peptides with immobilized Cu(II). This phenomenon probably involves secondary interaction mechanisms and is under further investigation. Nevertheless, each of our observations suggest that recognition of free metal ions by proteins, and perhaps peptides, is different from that of immobilized metal ions. The results presented here should enable the reinterpretation of other data generated with immobilized metal ions where the presence of free or competing metal ions was considered a negative or unknown influence

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