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# Metal ligand-induced alterations in the surface structures of lactoferrin and transferrin probed by interaction with immobilized copper(II) ions

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## ABSTRACT

We have evaluated immobilized Cu(II) ions as a potential site-directed molecular probe to monitor ligand-induced alterations in protein surface structures. Metal ion-induced alterations in the surface structures of different lactoferrins (human and porcine), transferrins (human and rabbit), and ovotransferrin (chicken) were examined. Although these 78 000-dalton glycoproteins are related gene products with similar overall structure and function, they differ greatly in the number and distribution of surface-exposed electron-donor groups thought to interact with Cu(II) ions. Each of these proteins interacted with immobilized Cu(II) ions through sites which are distinct from the two specific high affinity metal binding sites identified for iron. In both the presence and absence of bound iron, transferrins interacted more strongly with the immobilized Cu(II) ions than did lactoferrins; ovotransferrin interacted only weakly. Although iron binding *increased* the affinities of lactoferrins for immobilized Cu(II), iron binding *decreased* the affinities of transferrins and ovotransferrin for immobilized Cu(II) ions. Iron-saturated and iron-free lactoferrins were resolved by pH gradient elution, but only in the presence of 3 *M* urea; they were not resolved by imidazole affinity elution. Conversely, the iron-saturated and iron-free forms of transferrin were only separated by imidazole affinity elution. Urea did not influence the resolution of apo and holo ovotransferrins by imidazole . The differential effects of urea and imidazole suggest the participation of different types of surface electron-donor groups. The progressive site-specific modification of surface-exposed histidyl residues by carboxyethylation revealed several lactoferrin forms of intermediate affinity for immobilized iminodiacetate–Cu(II) ions. In summary, independent of species, the affinity for immobilized Cu(II) ions increased as follows: iron-saturated ovotransferrin < metal-free ovotransferrin < apolactoferrin < hololactoferrin < < diferric or holotransferrin < monoferric transferrin < apotransferrin. We have demonstrated the use of immobilized Cu(II) ions to distinguish and to monitor ligand-induced alterations in protein surface structure. The results are discussed in relation to protein surface-exposed areas of electron-donor groups.

## INTRODUCTION

Stationary phase-immobilized transition metal ions have been evaluated for their potential for peptide and protein purification [1–10]. These evaluations have resulted from the expectation that immobilized metal ions may interact with proteins and peptides in a site-specific manner [1,11]. A select group of model peptides and proteins have been used to demonstrate such interactions [3,4,9,12,13]. Little effort,

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however, has been focused on variations in the mobile phase that may modify the relative affinities (*i.e.*, specificity) with which immobilized metal ions interact with various solvent-exposed electron-donor groups on the surface of a protein [2,6,7,10]. We have found that variations in the concentration and type of salt [12] and the use of urea [6,7,10,14] or ethylene glycol can dramatically alter immobilized metal ion retention of peptides and proteins. We report here that such experimental approaches can greatly facilitate the use of high-performance stationary phases of immobilized metal ions to monitor ligand-induced alterations in protein surface structure.

Lactoferrins and transferrins were chosen for these investigations for several reasons. High-resolution structural data for rabbit serum holotransferrin and human lactoferrin (apo and holo forms) are now available [15–18]. Each of these proteins has a large number and variety of solvent-exposed electron-donor groups on its surface, so the ability to detect minor alterations is challenged. There is spectroscopic evidence to suggest that conformational changes are associated with occupancy of the two high-affinity metal-binding sites [19–21]. For lactoferrin, *in vitro* generation of the iron-free form by incubation at low pH has been shown to result in irreversible structural damage [19,21]. Because the apo and holo forms of lactoferrin have each been associated with a wide spectrum of different biological activities [21–29], separation of the apo and holo forms of lactoferrin is important. Except for a recent report from our laboratory [29], the resolution of apo and holo lactoferrins has not been reported previously by any means other than analytical-scale electrophoresis [30].

In this report, we demonstrate that (1) immobilized copper ions on high-performance stationary phases can be a sensitive probe of metal ligand-induced alterations in the surface structures of ovotransferrin, transferrins and lactoferrins, (2) these three subclasses of transferrin gene family products can be distinguished by their differential affinity for immobilized Cu(II) ions, (3) these proteins interact with immobilized Cu(II) ions through sites which are clearly distinct from the two specific high affinity metal binding sites identified for iron, (4) species-dependent variations in the amino acid sequence or bound carbohydrate moiety of these glycoproteins do not influence these findings, and (5) mobile phase constituents (*e.g.*, counter ions) and modifying reagents such as urea and imidazole differentially affect the resolution of the apo and holo forms of these proteins.

## MATERIALS AND METHODS

Ultra-pure urea was obtained from Bethesda Research Labs. (Gaithersburg, MD, U.S.A.). It was used immediately after it had passed through a mixed-function deionizing resin AG501-X8(D) from Bio-Rad (Richmond, CA, U.S.A.). Imidazole was obtained from Sigma (St. Louis, MO, U.S.A.) and used after treatment with Norit A charcoal to reduce ultraviolet (280 nm) absorption. Human and porcine lactoferrins were purified to homogeneity from colostrum by affinity chromatography on immobilized single-stranded DNA [29]. Metal ions were removed from the lactoferrins by prolonged incubation (4 h at 37°C) and dialysis (18 h at 4°C) against a low pH buffer (0.1 *M* sodium phosphate at pH 4.0–4.2 with 1 mg/ml ascorbic acid) containing 1 mg/ml deferoxamine mesylate (Sigma) as the iron chelator. Rabbit and human serum transferrins, and chicken ovotransferrin (substantially iron-free) were obtained from Sigma. To iron-saturate the lactoferrins, transferrins, or ovotransferrin, sodium

bicarbonate was added to a concentration of 0.1 *M*, followed by the addition of ferrous sulfate in 0.01 *M* HCl and incubation at 37°C for a minimum of 15 min. $^{59}Fe$ (DuPont NEN, Boston, MA, U.S.A.) was added, as indicated, to monitor iron dissociation and hololactoferrin or holotransferrin elution properties. Copper-saturated lactoferrins and transferrins were prepared by the addition of copper(II) sulfate in the presence of 0.1 *M* sodium bicarbonate. Excess metal ions were removed by dialysis. Metal ion saturation, loss of metal ions and/or metal ion transfer were 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 also analyzed by energy-dispersive X-ray fluorescence spectroscopy using a Kevex 0600 XRF spectrometer.

The TSK chelate 5PW (10 $\mu$m, 750 × 7.5 mm) columns, prepared with iminodiacetate functional groups [31], were a gift from Dr. Kato of Tosoh Manufacturing Co., Yamaguchi, 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, CT, U.S.A.) flow cell and a Corning (Ithaca, NY, U.S.A.) pH meter were used for continuous monitoring of the column effluent pH. After the metal-chelating stationary phase [immobilized iminodiacetate (IDA)] was washed with water, 50 m*M* copper sulfate in water was passed through the column. Excess metal ions were washed away with 0.1 *M* sodium acetate buffer at pH 3.5 containing 0.5 *M* NaCl (1 ml/min). Three different column equilibration and elution buffer systems were used. The immobilized Cu(II) columns were equilibrated with (i) 20 m*M* sodium phosphate (pH 7.0) containing 3 *M* urea and 0.5 *M* NaCl, (ii) 100 m*M* sodium acetate (pH 5.5 to 5.0) containing 0.5 *M* NaCl and 3 *M* urea (as indicated), or (iii) 20 m*M* sodium phosphate or 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.0) containing 0.5 *M* NaCl, 1 m*M* imidazole and 3 *M* urea (as indicated). A specific sample was loaded onto the column, which was then developed (1 ml/min) with a linear gradient of either (i) 0.1 *M* sodium phosphate, 0.5 *M* NaCl and 3 *M* urea from pH 7.0 to pH 3.6, or (ii) 0.1 *M* sodium acetate, 0.5 *M* NaCl and 3 *M* urea from pH 5.5 or 5.0 to pH 3.5, or (iii) 20 m*M* sodium phosphate or HEPES (pH 7.0) containing from 1 to 20 m*M* imidazole (± 3 *M* urea). The absorbance at 280 nm and pH were measured continuously. Imidazole gradient slopes were verified separately by monitoring absorbance at 230 nm. Where indicated, fractions of 1 min each were collected.

Diethylpyrocarbonate (Sigma) stock solution was prepared in anhydrous ethanol, and the concentration was determined immediately before use by adding an aliquot of the stock to a solution of known concentration of imidazole and measuring the absorbance at 240 nm on a Beckman DU-70 spectrophotometer. Aliquots of this stock solution of diethylpyrocarbonate were added to a known concentration of iron-saturated human lactoferrin at room temperature such that the final molar ratio of diethylpyrocarbonate to lactoferrin was equal to the values given in Fig. 5. No further alterations in lactoferrin retention times were observed after incubation for 30 to 40 min with the stated concentration of diethylpyrocarbonate.

Lactoferrin and transferrin protein structures were evaluated using the program FRODO [32] (version 6.6 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, TX, U.S.A.) and the program ACCESS [33] (version 2.0 by B.

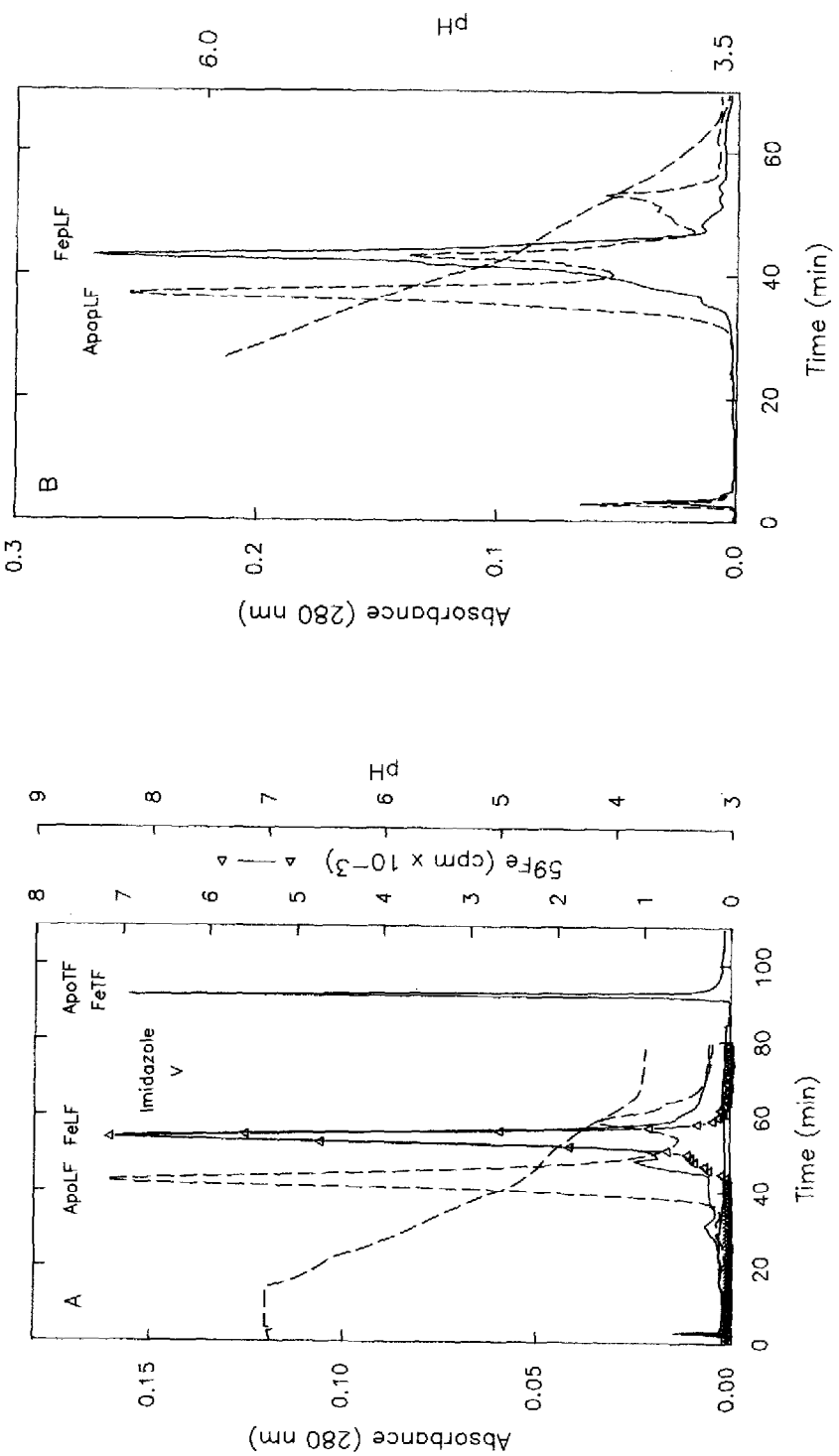



Fig. 1. Separation of apolactoferrin (dashed line) and iron-saturated human (A) and porcine (B) lactoferrins (solid line) on IDA–Cu(II) columns with a phosphate-buffered gradient of decreasing pH in the presence of 3 *M* urea. Both apotransferrin and iron-saturated transferrin were eluted only upon introduction of 20 m*M* imidazole (imidazole-labeled arrow). UV absorbance was monitored at 280 nm. The elution of iron-saturated human lactoferrin was determined by measuring protein-bound $^{59}Fe$ radioactivity (open triangles). **LF** = lactoferrin; **TF** = transferrin.

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. The refined coordinates for human lactoferrin [15–17] were generously provided by Dr. Edward N. Baker and colleagues at Massey University, New Zealand. The coordinates for diferric rabbit serum transferrin [18] were kindly provided by Dr. Peter Lindley at Birbeck College, University of London, U.K.

## RESULTS

The metal-free forms of ovotransferrin, transferrins, and lactoferrins were found to vary in the strength of their interaction with immobilized Cu(II) ions. Furthermore, the alteration(s) in protein surface structure that occurs upon free metal ion (iron) occupation of the two specific high affinity metal binding sites was apparent with IDA–Cu(II) affinity chromatography. The iron-saturated form of lactoferrin interacted with the immobilized Cu(II) ions with an affinity even higher than that of the iron-free form. Fig. 1A demonstrates the resolution of the metal-free and iron-saturated forms of human lactoferrin. We found that this resolution was only possible with high-performance IDA–Cu(II) columns eluted with a decreasing pH gradient in the presence of 3 *M* urea. Regardless of buffer type (*e.g.*, phosphate or acetate), urea was necessary to achieve the resolution of apo and hololactoferrins. Fig. 1A also illustrates the greatly increased affinity of human transferrin for IDA–Cu(II) relative to human lactoferrin.

Apotransferrin and iron-saturated transferrin, although tightly bound to IDA–Cu(II), were neither eluted nor resolved under conditions which permitted the resolution of apolactoferrin from hololactoferrin (Fig. 1A). The iron-free and iron-saturated forms of transferrin were eluted together by displacement with imidazole, after completion of the descending pH gradient.

The metal ligand (iron)-induced increase in the affinity of lactoferrin for immobilized Cu(II) ions was independent of species; similar results were obtained for human and porcine lactoferrins. Fig. 1B shows that the affinity (elution position) of iron-free porcine lactoferrin was less than that of iron-saturated porcine lactoferrin. Approximately 25–30% of the isolated (neat) porcine colostral whey lactoferrin was found to be iron-saturated upon spectroscopic analysis. The presence of this iron-saturated lactoferrin fraction in our purified porcine lactoferrin preparation was indicated by its co-elution with porcine lactoferrin which had been fully saturated with hiron *in vitro* before chromatography (Fig. 1B; solid line). The degree of bound iron in all fractions was routinely verified by spectroscopic analyses as described in Materials and Methods.

Fig. 2 demonstrates that human lactoferrin and human transferrin could also be separated on the IDA–Cu(II) affinity column using an acetate-buffered pH gradient. Again, however, the metal-free and iron-saturated forms of human lactoferrin could only be resolved using the acetate pH gradient protocol if 3 *M* urea was present (Fig. 2B). In the presence of 3 *M* urea, the lactoferrins and transferrins were resolved *within* the acetate pH gradient; the apo and holo forms of human transferrin, however, were still not separated. At the pH required for transferrin elution (pH 4), iron is readily released from transferrin [21]. This effect was confirmed by spectral analyses of the

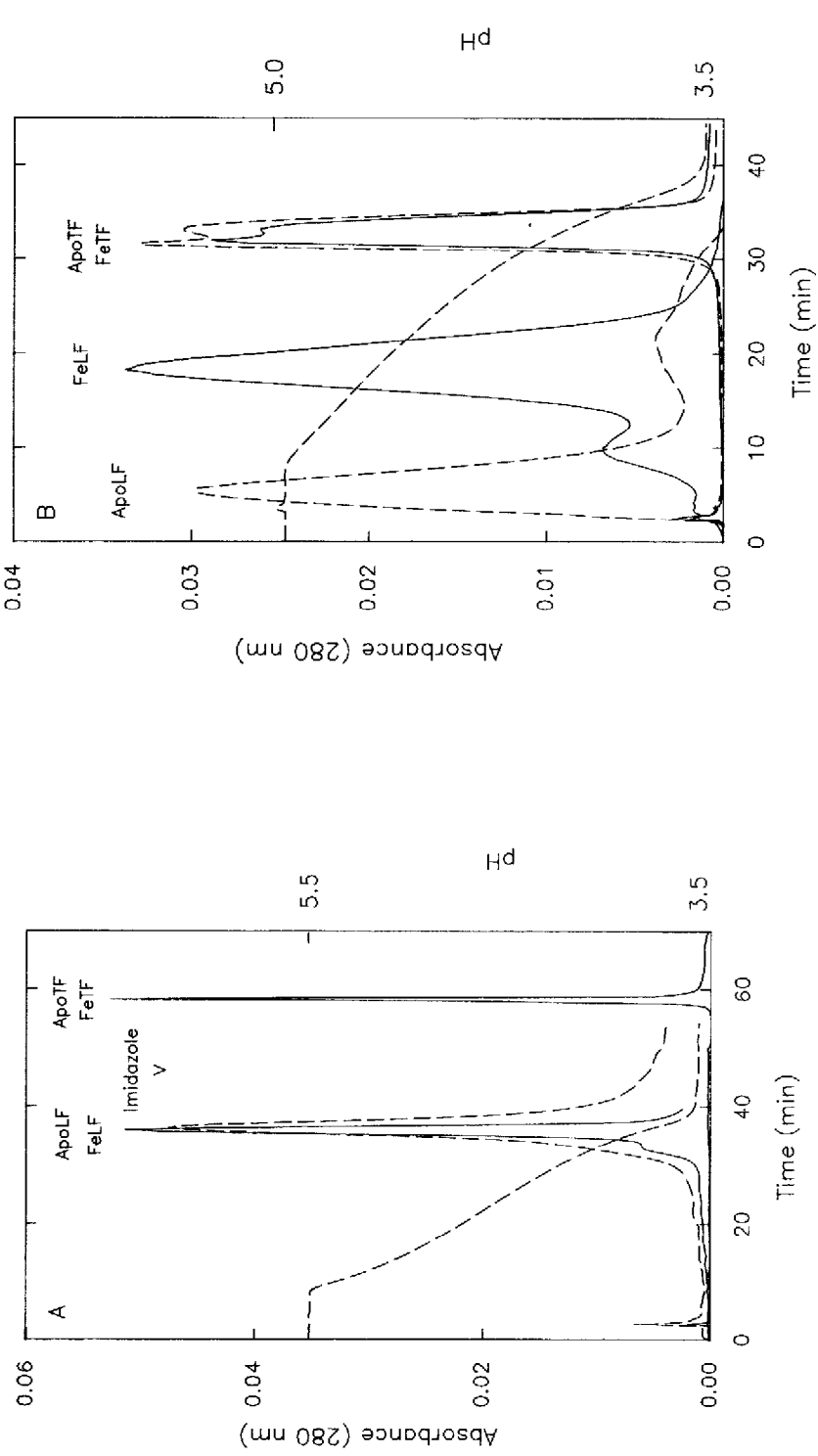



Fig. 2. Separation of the iron-free (dashed line) and iron-saturated (solid line) forms of human lactoferrin and human transferrin on IDA–Cu(II) affinity columns using an acetate buffer pH gradient elution protocol in the absence (A) and presence (B) of 3 *M* urea. In the absence of urea, both apotransferrin and iron-saturated transferrin were eluted only upon introduction of 20 m*M* imidazole (imidazole-labeled arrow).

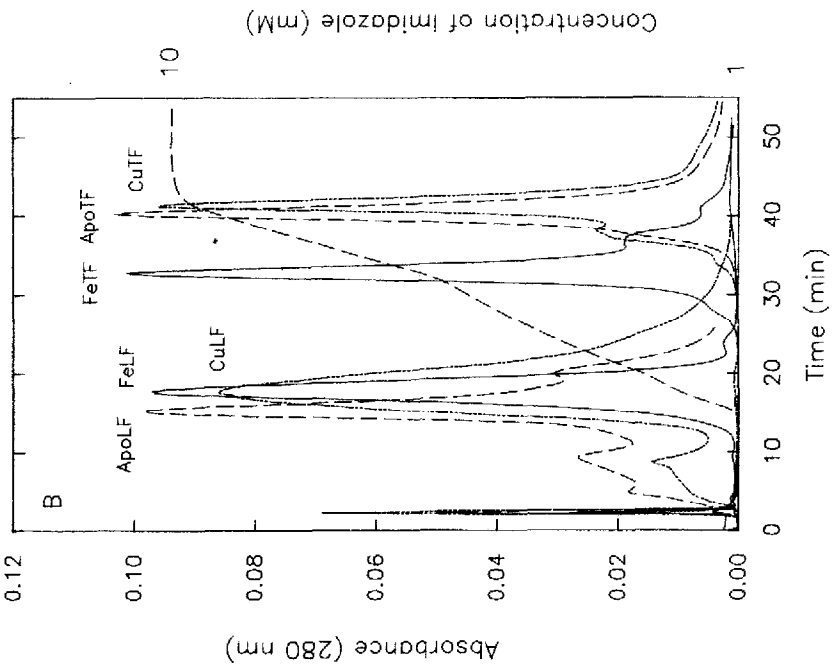
transferrin peak after pH gradient elution from the immobilized Cu(II) affinity column. Thus, it was not possible to resolve the apo and holo forms of transferrin by pH gradient elution under any of the conditions evaluated.

The elution profiles illustrated in Fig. 3A show that an increasing concentration gradient of imidazole (1–10 m*M*) was able to resolve the iron-free and iron-saturated forms of human transferrin at neutral pH (to preserve bound iron in the case of holotransferrin). The copper-saturated transferrin was not completely resolved from the iron-free (apo) form. In contrast, the metal-free, copper-saturated, and iron-saturated forms of human lactoferrin were not separated by the imidazole gradient elution protocol. Even in the presence of 3 *M* urea, only the apo form was slightly resolved from the metal-saturated forms (Fig. 3B). These results made it apparent that iron binding had opposite effects on the affinities of human lactoferrin and human transferrin for the immobilized Cu(II) ions: it *increased* lactoferrin affinity, but *decreased* transferrin affinity. Regardless of the elution protocol used, however, each form of transferrin (*i.e.*, apo and holo) demonstrated a higher affinity for immobilized Cu(II) ions than did any form of lactoferrin. To evaluate the species specificity of this observation, the effects of bound iron on human transferrin affinity for immobilized Cu(II) ions were confirmed with rabbit serum transferrin. Fig. 3C compares the elution properties of human and rabbit serum transferrins before and after saturation with iron.

Fig. 4 demonstrates that chicken ovotransferrin has a much weaker, but detectable, affinity for immobilized Cu(II) ions. Nevertheless, metal ligand (iron)-dependent alterations in ovotransferrin affinity for IDA–Cu(II) ions were clearly evident. Like the iron-saturated forms of human and rabbit transferrins, iron-saturated ovotransferrin showed a decreased affinity for immobilized Cu(II) ions. The metal ligand-dependent decrease in affinity for IDA–Cu(II) ions was observed in both the absence (Fig. 4A) and presence (Fig. 4B) of 3 *M* urea. In fact, the weak affinity of iron-free ovotransferrin for immobilized Cu(II) ions was unaffeced by the inclusion of urea.

To investigate the specific surface residues responsible for these observations, a relatively site-specific chemical modifying reagent was examined. The human lactoferrin elution profiles shown in Fig. 5 demonstrate the progressive alteration in hololactoferrin affinity for IDA–Cu(II) ions after carboxyethylation with increasing molar ratios of diethylpyrocarbonate. Multiple intermediate and low affinity interactions remained evident even after modification with a 10-fold molar excess of diethylpyrocarbonate. If the concentration of diethylpyrocarbonate was increased another 10-fold, lactoferrin interaction with the immobilized Cu(II) was eliminated.

Despite their similarity in size (78 000 dalton) and overall tertiary structure, an examination of the surface structures of rabbit serum holotransferrin and human lactoferrin (both apo and holo forms) revealed widely different local concentrations and distributions of amino acid residues, including electron-donor groups capable of interaction with immobilized transition metal ions. The program ACCESS [33] was used to calculate the degree to which specific residues were solvent-accessible, that is, able to achieve contact with a spherical probe the diameter of a water molecule (1.4 Å). We evaluated closely those residues already known or proposed to interact with immobilized transition metal ions (*i.e.*, His, Cys and Trp) [1,11–13]. Although several surface-exposed sulfur atoms were evident, neither protein contains reduced sulf-


A
ApoLF
FeLF
CuLF
FeTF
ApoTF
CuTF
Absorbance (280 nm)
0.10
0.05
0.00
0
20
40
60
Time (min)
1
10
Concentration of Imidazole (mM)


B
ApoLF
FeLF
CuLF
FeTF
ApoTF
CuTF
Absorbance (280 nm)
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0
10
20
30
40
50
Time (min)
1
10
Concentration of Imidazole (mM)

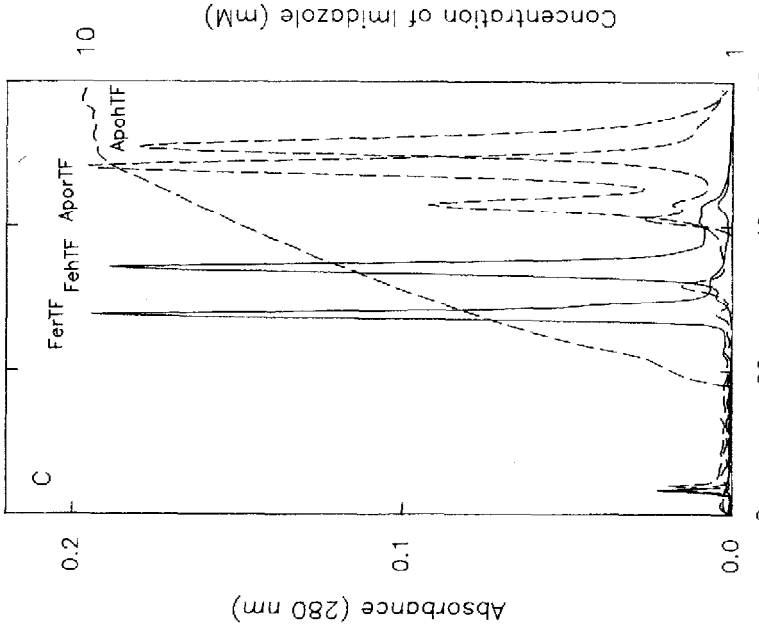



Fig. 3. Separation of iron-free (dashed line), iron-saturated (solid line), and copper-saturated (dashed/dotted line) lactoferrins and transferrins on an IDA–Cu(II) affinity column using an imidazole elution gradient in the absence (A and C) and presence (B) of 3 *M* urea. (A) and (B) illustrate results obtained with human lactoferrin (LF) and human transferrin (TF). (C) shows a comparison of results obtained with human serum transferrin (hTF) and rabbit serum transferrin (rTF). The Fe and Apo prefix to these abbreviations designates iron-saturated and metal-free transferrins, respectively.

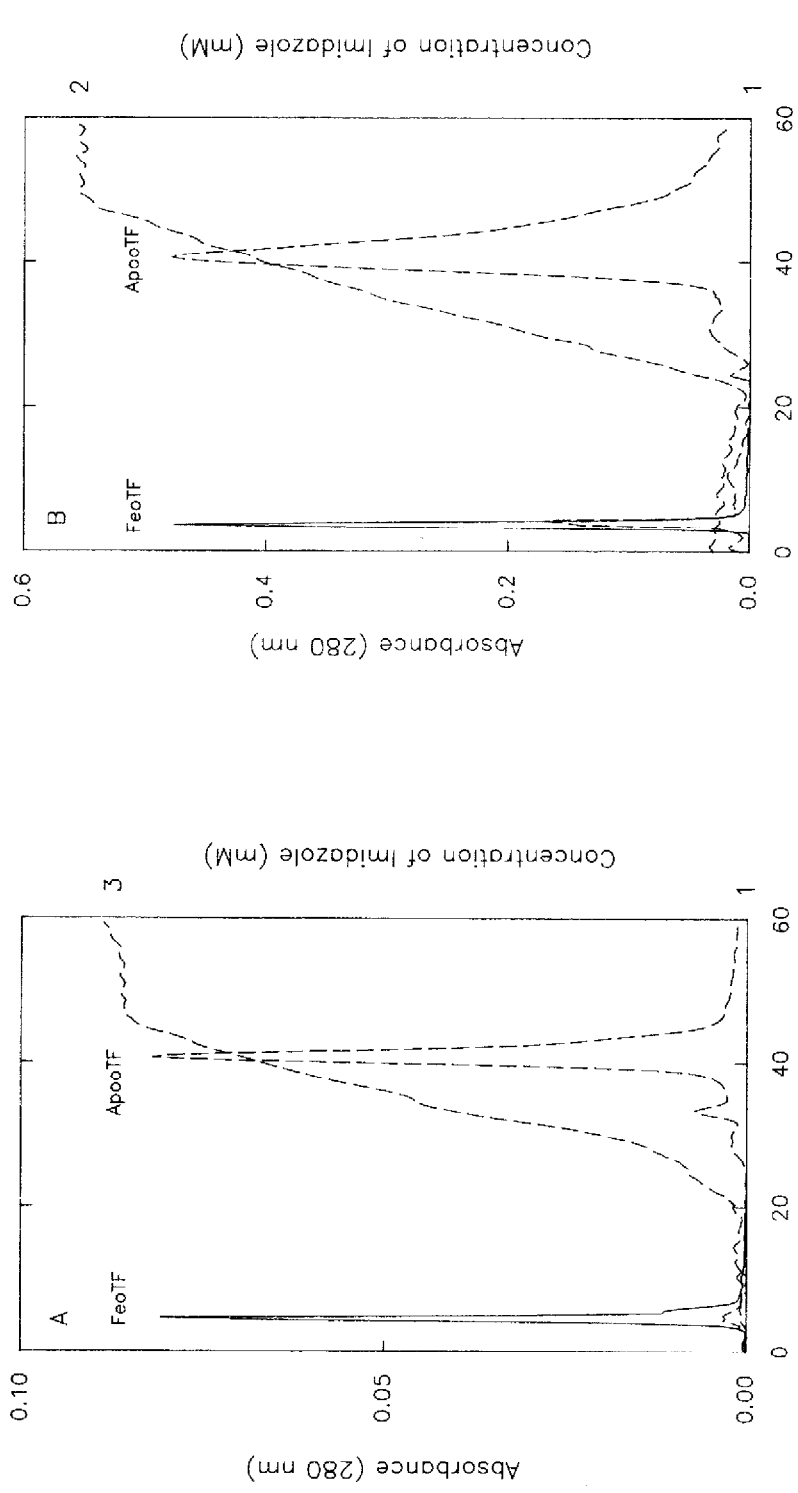



Fig. 4. Resolution of metal-free (Apo; dashed line) and Fe(II)-saturated (Fe; solid line) chicken ovotransferrins (oTF) on columns of IDA–Cu(II) using a shallow imidazole elution gradient (dashed line) in the absence (A) and presence (B) of 3 *M* urea.

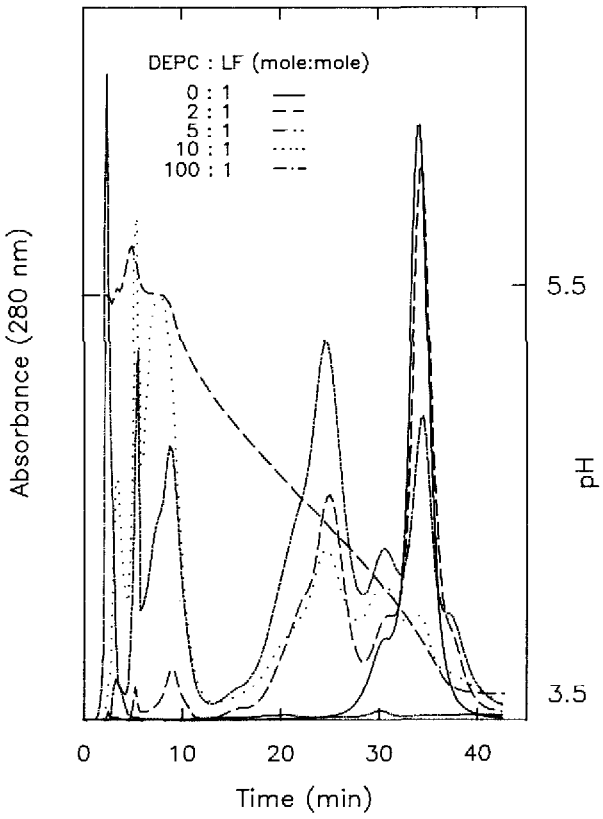



Fig. 5. Interaction of human hololactoferrin (LF) with immobilized Cu(II) ions after carboxyethylaton with increasing molar ratios of diethylpyrocarbonate (DEPC). The pH gradient elution protocol was as described in Fig. 2A.

hydryl groups. Lactoferrin and transferrin each contain two specific groups of metal binding residues within (approximately 1.0 nm from the molecular surface) the "iron-binding" clefts of their N-lobes and C-lobes. The specific metal-binding residues in the N-lobe of lactoferrin (Asp 60, Tyr, 92, Tyr 192 and His 253) and the C-lobe of lactoferrin (Asp 395, Tyr 435, Tyr 528 and His 597) are similar to those in the N-lobe of transferrin (Asp 63, Tyr 95, Tyr 188 and His 249) and the C-lobe of transferrin (Asp 392, Tyr 426, Tyr 517 and His 585) [15,16]. One can infer from the data presented here, however, that the amino acid residues in the two "specific" metal-binding clefts did not participate in the interaction of lactoferrin or transferrin with immobilized Cu(II) ions. Therefore, other more exposed electron-donor groups were evaluated. The total surface area of several types of electron-donor groups on the surface of rabbit holotransferrin was significantly greater than that of the human hololactoferrin. The relative differences between hololactoferrin and holotransferrin in total exposed surface areas of Trp residues (129 *versus* 163 $Å^2$), Phe residues (585 *versus* 833 $Å^2$) and Tyr residues (313 *versus* 433 $Å^2$) are small compared to the difference in exposed His residues (328 *versus* 1011 $Å^2$). The effects of specific near-neighbor relationships, although likely to be important, have not yet been considered.

## DISCUSSION

This investigation addresses the general problem of identifying specific experimental probes to monitor ligand-induced alterations in the surface structure of proteins. A primary concern was to identify probes that could also be used to achieve actual separation of such protein conformers. Stationary phase immobilized ligands specific for individual residues or domains are likely candidates for these purposes. Immobilized transition metal ions have already received considerable attention in this regard [1–4]. Surface-exposed histidine, cysteine, and tryptophan residues have been the primary focus of attention as the electron-donor groups likely to be responsible for protein and peptide interactions with immobilized transition metal ions [1,3,9,12]. However, the use of immobilized metal ions to monitor and exploit ligand-induced alterations in protein surface structures has not yet been examined.

In the case of lactoferrin, iron-binding is thought to initiate (or stabilize) a 53° inward domain rotation toward a more compact state [17]. It seemed likely that this ligand-induced alteration in surface structure might result in decreased interactions with immobilized metal ions. Thus, the results with lactoferrin were surprising. We have found that both Cu(II)-saturated lactoferrin and Fe(II)-saturated lactoferrin were bound to immobilized Cu(II) ions even more tightly than the metal-free or so-called apolactoferrin. We must conclude from these data that the interaction of lactoferrin with either Cu(II) or Fe(II) ions increases either the surface exposure or reactivity of residues responsible for the interaction with immobilized Cu(II) ions. We can conclude further that the two specific metal ligand binding sites are *not* contributing to the interaction of the metal ion-saturated lactoferrin forms with immobilized Cu(II) ions. The fact that apolactoferrin collected from the IDA–Cu(II) column could also be specifically labeled with two atoms each of either iron or copper ions provides additional evidence for our conclusion.

The effects of iron binding on transferrin and ovotransferrin affinity for immobilized Cu(II) ions were quite opposite the effects of bound iron on lactoferrin affinity for IDA–Cu(II). Iron binding decreased significantly the affinity of transferrin and ovotransferrin for immobilized Cu(II) ions. Iron-saturated transferrins were, however, still bound to IDA–Cu(II) with considerable affinity (higher affinity than any from of lactoferrin). Transferrin saturated with Cu(II) ions actually appeared to have an increased affinity for immobilized Cu(II) ions. Therefore, in contrast to recent conclusions presented elsewhere [34], the specific metal-binding ligands in the N- and C-lobes that are responsible for high affinity iron binding are *not* those involved in transferrin interaction with immobilized Cu(II) ions. Overall, the affinities of the ovotransferrin, lactoferrin, and transferrin for immobilized Cu(II) ions were found to increase as follows: iron-saturated (holo) ovotransferrin $<$ metal-free (apo) ovotransferrin $<$ apolactoferrin $<$ hololactoferrin $<<$ iron-saturated transferrin $<$ monoferric transferrin $<$ metal-free or apotransferrin. These findings were predicted in part by the large differences in surface-exposed areas of electron-donor groups (transferrins $>$ lactoferrins).

Through an inspection of the high-resolution structure of human apolactoferrin [17], two points can be made regarding lactoferrin affinity for immobilized metal ions. The quantity of solvent-exposed electron-donor groups previously thought to interact with immobilized metal ions (specifically His residues) on the surface of lactoferrin is

either unaffected or is actually *decreased* in the presence of bound iron, because bound iron stabilizes the closed (more compact) N-lobe and C-lobe configurations. Yet, we found that iron binding *increased* lactoferrin affinity for immobilized Cu(II) ions. If the number and area of surface-exposed immobilized metal ion binding sites is not changed, perhaps internal near-neighbor effects, altered by iron binding, act to increase the affinity of exposed groups for immobilized Cu(II) ions. In the case of transferrin, our data predict the opposite since iron binding decreases transferrin affinity for immobilized Cu(II) ions. These predictions await confirmation by structural solutions to the apo form of transferrin.

There were species-dependent deviations in lactoferrin and transferrin affinity for immobilized Cu(II) ions. Yet, the differences in the amino acid sequence and carbohydrate moieties for the two different transferrins (human and rabbit) and lactoferrins (human and porcine) evaluated did not influence the observed positive effects (for each lactoferrin) and negative effects (for each transferrin) of bound ligand (iron) on affinity for immobilized Cu(II) ions. The effects of altered carbohydrate composition (*e.g.*, recombinant lactoferrins) or carbohydrate removal (*e.g.*, deglycosylation) have not yet been evaluated in detail.

When the electron-donor groups on a protein surface are sufficiently solvent-exposed, mobile phase constituents (including buffer components and free metal ions) may influence their affinity or capacity to interact with immobilized metal ions. The use of mobile phase modifying reagents to promote selective protein recognition of immobilized ligands has been discussed (*e.g.*, ref. 35). The *differential* effects of urea and imidazole on the resolution of lactoferrin and transferrin in their apo and holo forms were clearly evident and suggest the participation of different types of surface electron-donor groups. In contrast to relatively simpler "one-site" protein models (*e.g.*, lysozyme), multiple types (affinities) of interactions exist between lactoferrin and IDA-immobilized Cu(II) ions [36]. Analyses of equilibrium binding data, performed as described previously [36,37], confirmed the contrasting influence of urea and imidazole on the interaction of these proteins with immobilized Cu(II) ions (data not shown). Much more work is needed to clarify the role of solvent on protein interactions with immobilized metal ions.

The site-specific carboxyethylation of lactoferrin with diethylpyrocarbonate did not abolish completely its affinity for immobilized Cu(II) ions until the molar ratio of diethylpyrocarbonate to surface-exposed histidyl residues was greater than 14:1. While histidine modification is most likely under these conditions, the actual number and type of surface residues modified has not been verified. We have also found that N-hydroxysuccinimide esters, known to react with primary amines (*e.g.*, lysine residues or amino terminus), can be used to eliminate the affinity of transferin for immobilized transition metal ions (unpublished observation). Thus, the mechanism(s) by which urea and imidazole differentially influence the reaction of specific residues with the immobilized metal ions remains unknown.

The effects of excess free metal ions in the mobile phase were negligible. Previous investigations [36] have shown that the presence of added free Cu(II) ions did not affect the chromatographic behavior of iron-saturated lactoferrin on affinity columns of immobilized Cu(II). Equilibrium binding experiments confirmed the absence of detectable alterations in lactoferrin affinity for immobilized Cu(II) ions even in the presence of 100 $\mu M$ Cu(II) [36]. These data demonstrate that protein surface binding

sites for free Cu(II) ions and IDA-immobilized Cu(II) ions are functionally distinct, that is, individual metal ion ligand binding sites on the surface of these proteins are not necessarily those that determine stable interactions with immobilized metal ions.

The unusually high affinity of transferrin (and lactoferrin) for immobilized Cu(II) ions must result from very effective and (or) very extensive protein surface–stationary phase interactions. Because of their distribution over the protein surface, and in the absence of extensive protein denaturation at the immobilized Cu(II) ion stationary phase, it seems unlikely that all of the electron-donor residues (*e.g.*, His residues) on the surface of lactoferrin or transferrin are participating in the immobilization event. Nevertheless, relatively distant attachment (*i.e.*, metal-binding) sites on a protein surface may interact with separate immobilized metal ions to create a macromolecular chelation (macrochelation) event (see Fig. 1 of ref. 37). This multi-point macrochelation mechanism results in a high affinity interaction event, even though the separate contribution by each individual residue may be relatively weak. This view is separate and distinct from the possibility of high affinity microchelation events which may occur when localized (vicinal) clusters of two or more surface-exposed residues participate in the interaction (microchelation) with a single immobilized metal ion.

Our results may have broader implications. Intracellular metal ions are associated with protein surfaces. These interactions remain largely uncharacterized. Protein surface-immobilized metal ions may influence macromolecular recognition events. The potential sensitivity of surface immobilized Cu(II) ions to distinguish and monitor ligand-induced alterations in protein surface structure has now been demonstrated *in vitro*. These findings suggest potential control mechanisms which may be operational *in vivo*. Given what we expect may be a general role for metal ions in the regulation or facilitation of biopolymer interactions [38], stationary phase immobilized metal ions present a useful model system for the further investigation of surface-immobilized metal ions in macromolecular recognition events.

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