Application of Immobilized Metal Ion Chelate Complexes as Pseudocation Exchange Adsorbents for Protein Separation†

Michael Zachariou and Milton T. W. Hearn*

Centre for Bioprocess Technology, Department of Biochemistry and Molecular Biology, Monash University, Wellington Road, Clayton, Victoria, Australia 3168

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ABSTRACT: The interactions of horse muscle myoglobin (MYO), tuna heart cytochrome *c* (CYT), and hen egg white lysozyme (LYS) with three different immobilized metal ion affinity (IMAC) adsorbents involving the chelated complexes of the hard Lewis metal ions Al^{3+}, Ca^{2+}, Fe^{3+}, and Yb^{3+} and the borderline Lewis metal ion Cu^{2+} have been investigated in the presence of low- and high-ionic strength buffers and at two different pH values. In contrast to the selectivity behavior noted with buffers of high ionic strength, with low-ionic strength buffers, these three proteins interact with the hard metal ion IMAC adsorbents in a manner more characteristic of cation exchange behavior, although in contrast to the cation exchange chromatography of these proteins, as the pH value of the elution buffer was increased, the retention also increased. The selectivity differences observed under these conditions appear to be due to the formation of hydrolytic complexes of these immobilized metal ion chelate systems involving a change in the coordination geometry of the *im*-M^{n+}−chelate at higher pH values. The experimental observations have been evaluated in terms of the effective charge on the immobilized metal ion chelate complex and the charge characteristics of the specific proteins.

Immobilization of chelating compounds onto solid matrices was introduced as a separation procedure in 1948 by Meinhardt (1948), although aspects of the interaction of metal ions with poorly characterized chelating substances impregnated into paper can be traced back at least to 1855 to the pioneering studies of Runge (1855). During the period from 1950, the high selectivity offered by multidentate compounds, particularly toward metal ions, resulted in immobilized chelate-based adsorbents rapidly gaining popularity in industry (Sahni & Reedijk, 1985). In 1974, a cation exchanger equilibrated with Al^{3+} ions was used (Shankar & Joshi, 1974) to fractionate RNA while 8-hydroxyquinoline, covalently attached to an agarose support and complexed with Zn^{2+} ions, was also used to isolate metalloproteins (Evenson & Parker, 1974). Subsequently, the use of immobilized metal ion chelate complexes was significantly expanded by the pivotal investigations of Porath and his co-workers with immobilized iminodiacetic acid (*im*-IDA)[1] and close analogues such as tricarboxyethylenediamine (TED) as the chelating compound for the isolation of proteins, leading to the introduction by this research group of the descriptive term coined for this separation technique; "immobilized metal ion affinity chromatography" (IMAC) (Porath et al., 1975, 1983; Porath & Olin, 1983; Porath, 1990). Various applications of IMAC with borderline Lewis metal ions have been reviewed in detail in recent years (Arnold, 1991; Sulkowski, 1988; Wong et al., 1991). Currently, investigators frequently favor the use of IDA as the chelating agent of choice with covalent attachment of this compound either to a soft gel matrix such as cross-linked agarose (Porath, 1990; Sulkowski, 1988; Wong et al., 1991) or alternatively to a chemically modified inorganic matrix such as silica or zirconia (Wirth et al., 1993), with subsequent complexation of borderline Lewis metal ions [as defined by Pearson (1990)] such as Cu^{2+}, Ni^{2+}, or Zn^{2+}. Numerous proteins with surface accessible histidine, tryptophan, or cysteine residues have been purified with these so-called borderline *im*-M^{n+}−IDA adsorbents (Kagedal, 1989; Arnold, 1991; Wong et al., 1991; Sulkowski, 1988).

In contrast to the extensive literature on the use of borderline *im*-M^{n+}−chelate complexes, remarkably few systematic studies on the interactive behavior of proteins with different types of immobilized hard Lewis M^{n+}−chelate complexes, particularly with low-ionic strength eluents, have been described. As a consequence, the contribution that hard metal ions such as Fe^{3+} or Ca^{2+} can make to the selectivity of protein binding in IMAC cannot yet be accurately anticipated. Furthermore, the contribution to protein selectivity made by the immobilized chelating ligand itself, as part of the hard *im*-M^{n+}−chelate complex, under conditions that can enhance or reduce electrostatic interactions with these hard metal ions, has also not yet been well-documented. In the present investigations, results are described which reveal the binding behavior of three proteins in the presence of the hard Lewis metal ions Al^{3+}, Ca^{2+}, Fe^{3+}, and Yb^{3+}, and the borderline Lewis metal ion Cu^{2+} as a control cation, using *im*-IDA (Figure 1), *im*-8-HQ (Zachariou & Hearn,

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* To whom correspondence should be addressed. Fax: 61+3+905-5882. Telephone: 61+3+905-3720. E-mail: milton.hearn@med.monash.edu.au.

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[1] Abbreviations: CYT, tuna heart cytochrome *c*; 8-HQ, 8-hydroxyquinoline; IDA, iminodiacetic acid; *im*-, immobilized; *im*-8-HQ, immobilized 8-HQ adsorbent; *im*-IDA, immobilized IDA adsorbent; *im*-OPS, immobilized *O*-phosphoserine adsorbent; *im*-M^{n+}−chelate, immobilized metal ion chelate adsorbent; *im*-M^{n+}−8-HQ, immobilized metal ion 8-HQ adsorbent; *im*-M^{n+}−IDA, immobilized metal ion IDA adsorbent; *im*-M^{n+}−OPS, immobilized metal ion OPS adsorbent; IMAC, immobilized metal ion affinity chromatography; LYS, hen egg white lysozyme; MYO, horse muscle myoglobin; OPS, *O*-phosphoserine; TED, tricarboxyethylenediamine.

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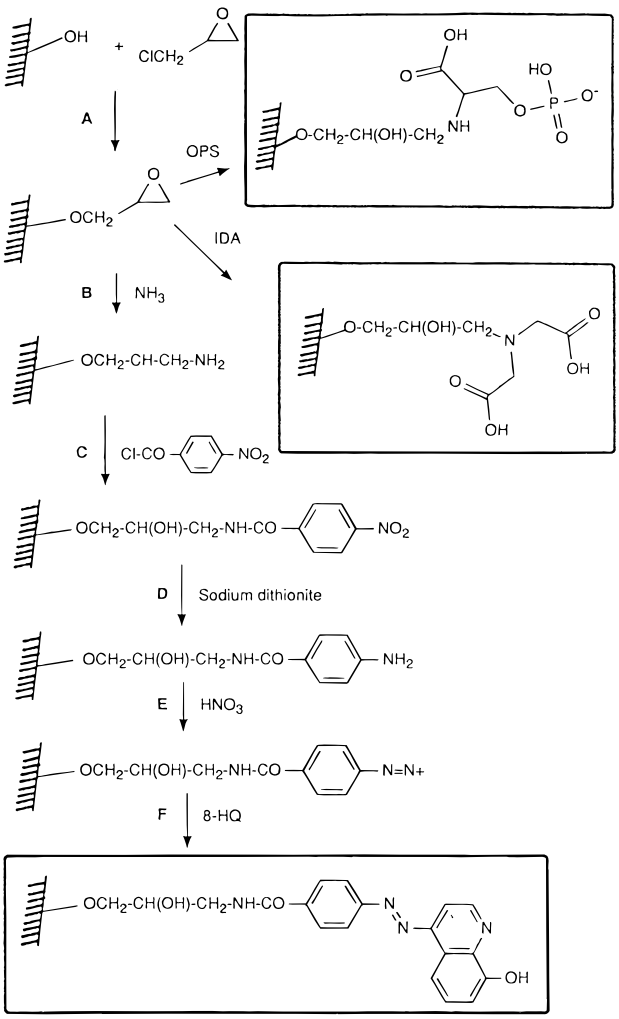

FIGURE 1: Diagramatic representation of the structure and the synthetic route used for the preparation of the immobilized *O*-phosphoserine (OPS), 8-hydroxyquinoline (8HQ), and iminodiacetic acid (IDA) on Sepharose CL-4B chelated to a hexacoordinate M^{n+} metal ion.

1992) (Figure 1), and the recently introduced ligand *im*-OPS (Zachariou et al., 1993) (Figure 1) as the chelating species at pH 5.5 and 8.0. The adsorption and elution behavior of three proteins (tuna heart cytochrome *c*, horse skeletal muscle myoglobin, and hen egg white lysozyme) at different ionic strengths and pH values with these *im*-M^{n+}−chelates has been examined in order to establish trends in relative affinities for the protein−*im*-M^{n+}−chelate complexes. This behavior was then compared to the binding and elution behavior of the same proteins with the conventional cation exchanger CM-Sepharose. The results indicate that the hard Lewis M^{n+}−chelate complexes show higher affinity and different selectivities for the test proteins when low-ionic strength buffers are used than when high-ionic strength buffers are employed. Moreover, when different types of *im*-chelate complexes are used with the same hard metal ion, the IMAC selectivities for these proteins were also significantly different over the same operational pH range.

MATERIALS AND METHODS

Source of Proteins and Reagents. Tuna heart cytochrome *c* (CYT), horse skeletal muscle myoglobin (MYO), and hen egg white lysozyme (LYS) were purchased from Sigma-Aldrich (St. Louis, MO) and repurified on the basis of methods described previously (Richards et al., 1994). Sepharose CL-4B and CM-Sepharose Fast Flow were purchased from Pharmacia Biotech AB (Uppsala, Sweden). Metal nitrate salts and all other reagents were purchased from Aldrich Chemical Co. (Castle Hill, Australia) and were of analytical grade purity unless otherwise stated.

Immobilization of Chelating Agents to Sepharose CL-4B. The immobilization of IDA onto epoxy-activated Sepharose CL-4B, washed extensively with 20 volumes of Milli-Q water, was carried out as described previously (Porath & Olin, 1983). The procedure described by Zachariou et al. (1993) was used for the immobilization of the OPS−Sepharose CL-4B which was then washed with 10 volumes of Milli-Q water, 5 volumes of 50 mM acetic acid (pH 4.0), and 10 volumes of Milli-Q water. The procedure described by Zachariou and Hearn (1992) was followed for the immobilization of 8-HQ onto epoxy-activated Sepharose CL-4B.

Elemental analyses of the immobilized chelating agents were carried out, with the OPS−Sepharose CL-4B adsorbents analyzed for their nitrogen and phosphorus content by Dairy Technical Services (Melbourne, Australia) and the IDA−Sepharose CL-4B and 8-HQ−Sepharose CL-4B adsorbents analyzed for their nitrogen content. The absolute error for the nitrogen analysis was 0.01% (w/w of the dry weight of the IMAC adsorbent), while that for the phosphorus analysis was 0.05% (w/w of the dry weight of the IMAC adsorbent).

Metal Ion Binding Studies. The various metal ions were loaded onto the *im*-chelate−Sepharose CL-4B as described previously (Zachariou & Hearn, 1992, 1995). Briefly, the chelating adsorbents were incubated with 10 mM $Fe(NO_3)_3$ or $Al(NO_3)_3$ or 50 mM $Ca(NO_3)_2$, $Cu(NO_3)_2$, or $Yb(NO_3)_3$ solutions for 30 min at 25 °C. The *im*-M^{n+}−chelates were then washed with 10 volumes of Milli-Q water and subsequently incubated with 10 volumes of 50 mM acetic acid containing 0.1 M KNO_3 (pH 4.0) (buffer A) for 10 min at 25 °C to remove any weakly bound metal ions. The *im*-M^{n+}−chelates were then rinsed with 10 volumes of Milli-Q water before the *im*-M^{n+}−chelates were incubated with the appropriate equilibration buffer and the suspension was degassed under vacuum for 30 min at 25 °C. The equilibration buffers used in these investigations were as follows: (i) buffer B, 30 mM Mes, 30 mM imidazole, and 0.005% (v/v) Brij-35, adjusted to pH 5.5 with HCl; and (ii) buffer C, 30 mM Hepes, 30 mM imidazole, and 0.005% (v/v) Brij-35, adjusted to pH 8.0 with NaOH. These buffers were also used for the protein binding studies described subsequently and were designed to have a constant ionic strength of about 0.06 M. The Mes and Hepes buffers were chosen not only for their good buffering capacities over the required pH range but also for their poor metal ion complexing abilities (Good et al., 1966). For the studies involving the equilibration/loading buffers, 3 mM imidazole was also included so as to quench any affinity that exposed histidine residues in the test proteins could have for the metal ions, in particular Cu^{2+} ions (Zachariou & Hearn, 1992, 1995). Brij-35 was included so as to minimize any nonspecific hydrophobic interactions of proteins with the adsorbent. The nonionic detergent Brij-35 does not contribute to the ionic strength or interfere in the IMAC interaction (Kagedal, 1989). The concentration of Brij-35 used was below its critical micelle concentration (Neugebauer, 1988). All buffers were degassed under vacuum before use.

Table 1: Amount of Immobilized Metal Ion per Mole of Immobilized Chelating Agent[a]

	OPS−Sepharose CL-4B		8-HQ−Sepharose CL-4B		IDA−Sepharose CL-4B	
	pH 5.5	pH 8.0	pH 5.5	pH 8.0	pH 5.5	pH 8.0
Al^{3+}	0.76 ± 0.01	0.78 ± 0.07	0.32 ± 0.01	0.30 ± 0.01	0.44 ± 0.01	0.46 ± 0.02
Ca^{2+}	0.07 ± 0.01	0.07 ± 0.01	0.06 ± 0.01	0.12 ± 0.01	0.10 ± 0.01	0.09 ± 0.01
Cu^{2+}	0.58 ± 0.02	0.60 ± 0.01	0.29 ± 0.02	0.33 ± 0.02	0.97 ± 0.03	0.93 ± 0.01
Fe^{3+}	1.08 ± 0.02	1.11 ± 0.04	0.73 ± 0.04	0.77 ± 0.02	1.04 ± 0.04	1.04 ± 0.03
Yb^{3+}	0.55 ± 0.03	0.50 ± 0.02	0.22 ± 0.02	0.21 ± 0.01	0.64 ± 0.01	0.66 ± 0.03

[a] Metal ion analysis on Sepharose CL-4B was carried out as described in Materials and Methods. The results represent the average moles of metal ion (M^{n+}) bound to the mole of immobilized chelating agent, with the standard error from the mean from duplicate experiments.

The *im*-M^{n+}−chelates were freeze-dried before being analyzed for metal ion content, as described elsewhere (Zachariou & Hearn, 1995). Analyses for metal ion content were carried out by atomic absorption spectroscopy using a Varian Spectra 300 instrument located at the Analytical Services Department (CSL, Parkville, Australia). The Yb^{3+} ion content was analyzed by means of flame emission spectroscopy, also performed at the Analytical Services Department (CSL, Parkville, Australia). All metal ion binding studies and metal ion content analyses were performed at least in duplicate. The moles of *im*-metal ion per mole of *im*-ligand (*M*) were calculated from $M = A/B$, where A = moles of *im*-metal ion per gram of dry Sepharose CL-4B and B = moles of *im*-chelator per gram of dry Sepharose CL-4B.

Chromatography Studies. The *im*-M^{n+}−chelates (1 mL), equilibrated with buffer B or C, were packed into Bio-Rad econocolumns. Aliquots of the test protein solutions, containing 16 nmol of each of the different proteins (CYT, LYS, and MYO as 1.0 mg/mL protein solutions), singly and as mixtures, were loaded onto the individual columns, which were then washed with 4 mL of the appropriate equilibration buffer. The breakthrough/wash volume was collected and labeled the "nonadsorbed" fraction. This experimental design approximates batch adsorption methods (Anspach et al., 1989), allowing the bound and free protein concentrations to be readily determined and the relative affinities and capacities of the *im*-M^{n+}−8-HQ, or *im*-M^{n+}−OPS adsorbents to be assessed for the different proteins. The volume ratio of *im*-M^{n+}−chelate to equilibrating buffer used for this column washing step was chosen to ensure that all of the protein that did not bind, or only weakly bound, was collected as the nonadsorbed fraction. Similar ratios of the volume of adsorbent to the volume of equilibrating buffer used for washing unbound proteins from the adsorbents have previously been employed (Scopes, 1984) to screen triazine dye affinity sorbents for enzyme isolation. The metal ion contents on the *im*-M^{n+}−chelates were chosen to be between 30 and 1300 times greater in molar terms than the amount of the loaded protein, so as to ensure that protein overload of the *im*-M^{n+}−chelate did not occur. The bound proteins were eluted from the *im*-M^{n+}−chelate by the addition of 3.2 mL of the following buffers: (i) buffer D, buffer B + 0.1 M NaCl (pH 5.5) for proteins which bound at pH 5.5; or (ii) buffer E, buffer C + 0.1 M NaCl (pH 8.0) for proteins which bound at pH 8.0. Once this bound fraction had been collected, the columns were then washed with a further 3.2 mL of the following buffers: (iii) buffer F, buffer B + 0.3 M NaCl (pH 5.5) for proteins which were still bound after the column was washed with buffer D; or (iv) buffer G, buffer C + 0.3 M NaCl (pH 8.0) for proteins which were still bound after the column was washed with buffer E. All elution experiments were done in duplicate. Protein analysis was achieved by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and quantitation by using an analytical high-performance liquid chromatography (HPLC) method with a J. T. Baker (Phillipsburg, NJ) Bakerbond C_8 reversed phase column attached to a Hewlett-Packard 1090 HPLC and 1040 diode array detector set at 215 and 400 nm wavelengths, as described elsewhere (Zachariou & Hearn, 1992; Zachariou et al., 1993).

RESULTS

In order to study the binding properties of the test proteins with the *im*-M^{n+}−chelates, variation of the parameters of pH and ionic strength was utilized. The proteins were chosen on the basis of their similar size, thus avoiding adsorption differences due to pore size effects with the IMAC adsorbents, the surface distribution of their charged amino acids, their relatively high isoelectric points, namely MYO (pI = 6.8), CYT (pI = 10.5), and LYS (pI = 11.3), and their content of histidine, aspartic acid, and glutamic acid residues. Bound proteins were eluted from the *im*-M^{n+}−chelates and the control cation exchange adsorbent, CM-Sepharose, using stepwise NaCl concentration increments, first with 0.1 M NaCl and then with 0.3 M NaCl.

Determination of Metal Ion Content and Chelating Ligand Contents

A combination of atomic absorption spectroscopy, flame emission spectroscopy, and elemental analysis was used to assess the metal ion and the chelating ligand contents of newly prepared samples of the various IMAC adsorbents. The results are summarized in Table 1. The ligand contents per gram of Sepharose CL-4B of *im*-IDA, *im*-8HQ, and *im*-OPS were 6.0×10^{-4} mol of IDA/g of dry Sepharose CL-4B, 3.3×10^{-4} mol of H-HQ/g of dry Sepharose CL-4B, and 4.0×10^{-4} mol of OPS/g of dry Sepharose CL-4B, respectively. Within the precision of the experimental measurements, it can be concluded that the ligand densities of the *im*-M^{n+}−8-HZ- and *im*-M^{n+}−OPS-based IMAC adsorbents (and their metal ion free counterparts) at pH 5.5 and 8.0 were similar. No significant ligand leakage was induced by these different pH conditions over extended periods of storage at 4 or 22 °C, i.e. 20 days plus. As a consequence, the observed differences in adsorption behavior of the three test proteins reflect differences in their selectivity and relative affinity preferences for the various IMAC-related adsorbents of similar ligand densities at the two pH conditions.

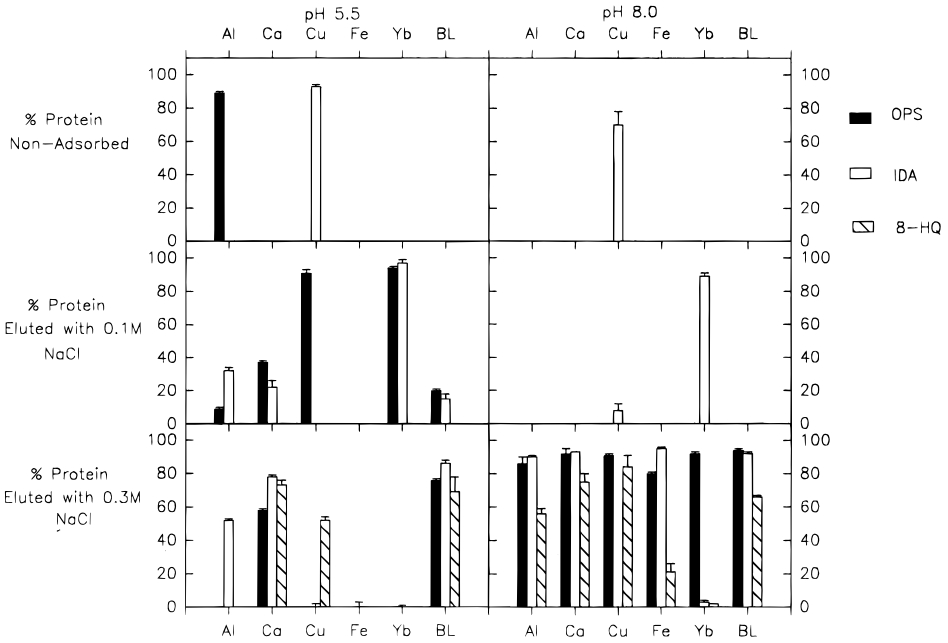

FIGURE 2: Binding and elution behavior of CYT on immobilized M^{n+} chelate Sepharose CL-4B. The adsorption, elution, and protein quantitation experiments were carried out as described in Materials and Methods. The results represent the average amount of protein recovered from duplicate experiments at the adsorption and elution steps, with the standard errors from the mean indicated as error bars.

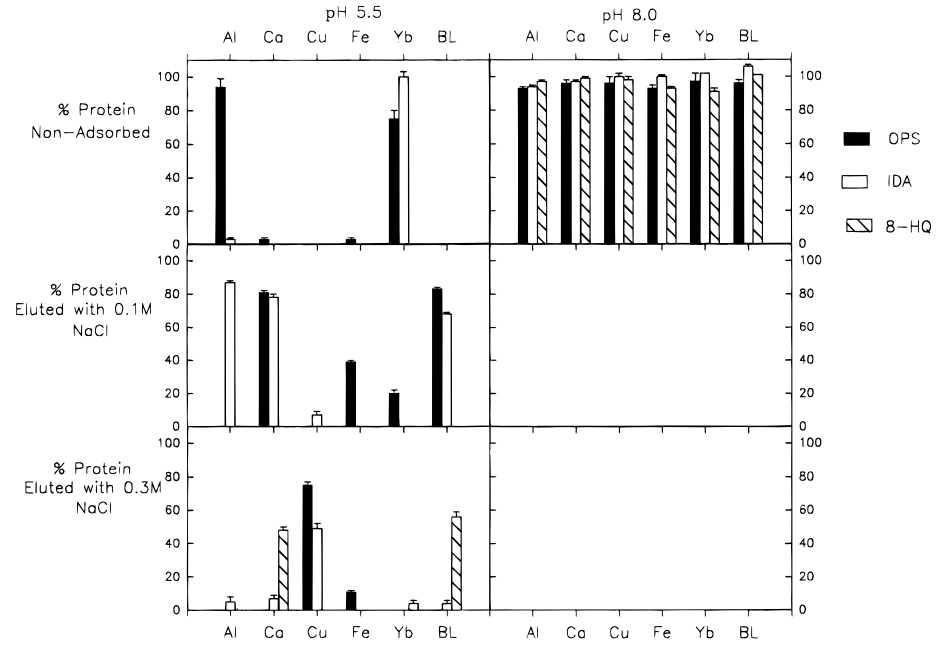

FIGURE 3: Binding and elution behavior of MYO on immobilized M^{n+} chelate Sepharose CL-4B. The adsorption, elution, and protein quantitation experiments were carried out as described in Materials and Methods. The results represent the average amount of protein recovered from duplicate experiments at the adsorption and elution steps, with the standard errors from the mean indicated as error bars.

Protein Binding Capacities and Relative Affinities for the Immobilized M^{n+}−Chelate and CM-Sepharose as a Function of pH

The adsorption and elution experiments described above allowed evaluation of the relative binding affinities of these proteins for the different *im*-M^{n+}−chelates and CM-Sepharose. The results from these experiments with MYO, CYT, and LYS and the *im*-M^{n+}−chelates and CM-Sepharose, at two different pH values, pH 5.5 and 8.0, are shown in Figures 2−5. In all cases at pH 8.0, MYO did not bind to any *im*-M^{n+}−chelate or to CM-Sepharose, while both CYT and LYS exhibited different binding behavior, depending on the particular type of *im*-M^{n+}−chelate. Overall, the various *im*-M^{n+}−chelate and CM-Sepharose at pH 8.0 showed the following order of relative binding affinities for these proteins: LYS > CYT ≫ MYO. For most of the *im*-M^{n+}−chelates equilibrated at pH 5.5, a similar trend in protein binding behavior was observed. However, several notable divergencies occurred in the selectivity order at pH 8.0. These exceptions are summarized in Table 2. For example, at pH 5.5, *im*-Cu^{2+}−OPS bound to MYO more strongly than to LYS, which was in turn bound more strongly than CYT. However, under the same pH 5.5 conditions, *im*-Fe^{3+}−OPS bound CYT more strongly than either LYS or MYO. Both *im*-Fe^{3+}−IDA and *im*-Cu^{2+}−IDA at pH 5.5 also showed deviations from the protein selectivity order seen at pH 8.0,

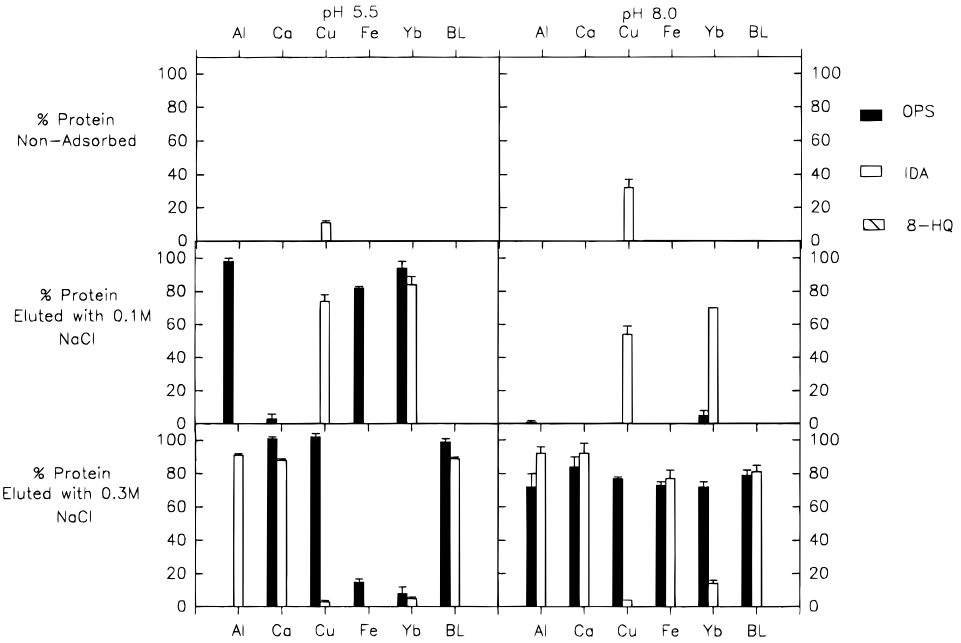

FIGURE 4: Binding and elution behavior of LYS on immobilized M^{n+} chelate Sepharose CL-4B. The adsorption, elution, and protein quantitation experiments were carried out as described in Materials and Methods. The results represent the average amount of protein recovered during duplicate experiments at the adsorption and elution steps, with the standard errors from the mean indicated as error bars.

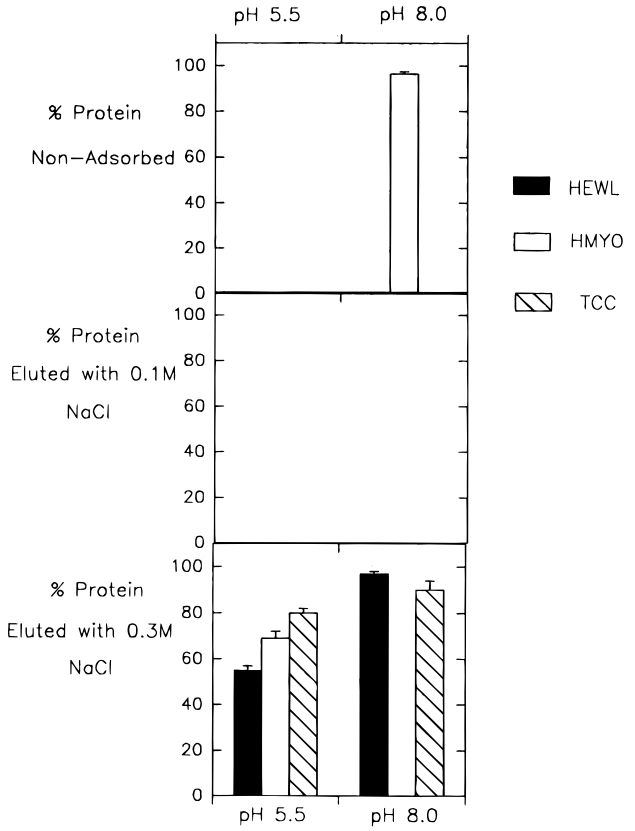

FIGURE 5: Adsorption and elution behavior of proteins on CM-Sepharose. The adsorption, elution, and protein quantitation experiments were carried out as described in Materials and Methods. The results represent the average amount of protein recovered from duplicate experiments at the adsorption and elution steps, with the standard errors from the mean indicated as error bars.

e.g. *im*-Cu^{2+}−IDA at pH 5.5 bound MYO more strongly than LYS which in turn was bound more strongly than CYT. In contrast, *im*-Fe^{3+}−IDA at pH 5.5 bound MYO more strongly than either LYS or CYT, but in this case, both these latter proteins exhibited similar relative affinities for this adsorbent. The *im*-Ca^{2+}−8-HQ and *im*-metal ion free 8-HQ were the only two *im*-8-HQ adsorbents which exhibited protein binding behavior with LYS > CYT ≫ MYO at pH 5.5. Moreover, *im*-Al^{3+}−8-HQ and *im*-Fe^{3+}−8-HQ very strongly bound the three proteins at pH 5.5, such that they could not be eluted with 0.3 M NaCl. Although *im*-Cu^{2+}−8-HQ also bound LYS and MYO at pH 5.5 with relatively high affinity, such that these two proteins could not be eluted with 0.3 M NaCl, CYT in comparison was readily eluted under these ionic strength conditions.

Table 2: Notable Divergencies in the Relative Protein Selectivity Order at pH 5.5 as a Function of Immobilized M^{n+}−Chelate Complex[a]

immobilized chelate−M^{n+}	relative protein affinity order at pH 5.5
OPS−Cu^{2+}	MYO > LYS > MYO
OPS−Fe^{3+}	TCC > LYS > MYO
IDA−Cu^{2+}	MYO > LYS > TCC
IDA−Fe^{3+}	MYO > LYS, TCC
8-HQ−Al^{3+}	LYS, TCC, MYO
8-HQ−Cu^{2+}	LYS, MYO > TCC
8-HQ−Fe^{3+}	LYS, TCC, MYO
8-HQ−Yb^{3+}	LYS, TCC > MYO
8-HQ−BL	LYS > MYO > TCC

[a] The results shown in this table illustrate the immobilized M^{n+}−chelate systems which exhibited a selectivity order different from the trend of LYS > CYT ≫ MYO observed at pH 8.0.

Protein Retention to Immobilized M^{n+}−Chelate Adsorbents as a Function of Metal Ion Content and Different pH Values

Additional comparisons of the experimental data permitted assessment of the relative selectivity differences between the *im*-M^{n+}−chelates (and CM-Sepharose) at the different pH values. Several noteworthy selectivity differences between the *im*-M^{n+}−chelates, the *im*-metal ion free chelates, and CM-Sepharose were evident. These results can be summarized as follows.

Table 3: Relative Immobilized Chelate Affinity Order for Protein as a Function of Metal Ion, at pH 5.5 and 8.0

	chelated metal ions				
protein	Al^{3+}	Ca^{2+}	Cu^{2+}	Fe^{3+}	Yb^{3+}
LYS at pH 5.5	8-HQ > IDA > OPS	8-HQ > IDA > OPS	8-HQ > OPS > IDA	8-HQ, IDA > OPS	8-HQ > IDA, OPS
CYT at pH 5.5	8-HQ > IDA > OPS	8-HQ > IDA > OPS	8-HQ > OPS > IDA	8-HQ, IDA, OPS	8-HQ > IDA, OPS
MYO at pH 5.5	8-HQ > IDA > OPS	8-HQ > IDA, OPS	8-HQ > OPS > IDA	8-HQ, IDA > OPS	8-HQ > OPS > IDA
LYS at pH 8.0	8-HQ > OPS > IDA	8-HQ > OPS, IDA	8-HQ > OPS > IDA	8-HQ > OPS, IDA	8-HQ > OPS > IDA
CYT at pH 8.0	8-HQ > OPS, IDA	8-HQ > OPS, IDA	8-HQ, OPS > IDA	8-HQ > OPS > IDA	8-HQ > OPS > IDA
MYO at pH 8.0	no binding	no binding	no binding	no binding	no binding

(A) Investigations with Immobilized IDA Adsorbents. At pH 5.5, only *im*-Fe^{3+}−IDA bound CYT more strongly than CM-Sepharose or the metal ion free (blank) *im*-IDA. At pH 5.5, CYT did not bind to *im*-Cu^{2+}−IDA and only weakly bound at pH 8.0. The relative affinities of CYT at pH 8.0 for the *im*-Al^{3+}−, *im*-Ca^{2+}−, and *im*-Fe^{3+}−IDA and the metal ion free IDA adsorbent were similar to those of CM-Sepharose. Similarly, with LYS, the adsorption results at pH 5.5 indicated that binding was strongest with *im*-Fe^{3+}−IDA followed by CM-Sepharose. In contrast to the results with CYT, LYS however bound to *im*-Cu^{2+}−IDA at pH 5.5. The adsorption results for LYS at pH 8.0 reveal that *im*-Fe^{3+}− and *im*-Al^{3+}−IDA and the metal ion free IDA adsorbents bound LYS more strongly than CM-Sepharose. Again, an increase in the pH of the adsorption buffer resulted in an increased affinity between the proteins and the *im*-M^{n+}−IDA. MYO did not bind to any *im*-M^{n+}−IDA at pH 8.0. At pH 5.5, *im*-Fe^{3+}−IDA bound MYO more strongly than CM-Sepharose, while the *im*-Ca^{2+}−, *im*-Al^{3+}−, and *im*-Yb^{3+}−IDA as well as the metal ion free adsorbents showed similar relative affinities with MYO.

(B) Investigations with Immobilized M^{n+}−OPS Adsorbents. Similar adsorption experiments confirmed that at pH 5.5 CYT bound to all the *im*-M^{n+}−OPS and *im*-metal ion free OPS. At pH 5.5, the *im*-Fe^{3+}−OPS bound CYT most strongly, followed by CM-Sepharose and then the metal ion free *im*-OPS, while the *im*-Al^{3+}−OPS bound CYT most weakly. At pH 8.0, however, all of the *im*-M^{n+}−OPS adsorbents bound CYT at least as strongly as CM-Sepharose, with the *im*-Fe^{3+}−OPS adsorbent again binding CYT most strongly. These results at pH 5.5 with CYT can be contrasted to the pH 5.5 experiments with LYS where CM-Sepharose bound this protein more strongly than any M^{n+}−OPS adsorbent. At pH 8.0, *im*-Fe^{3+}−, *im*-Ca^{2+}−, and *im*-Cu^{2+}−OPS and the *im*-metal ion free OPS bound LYS with higher relative affinities than CM-Sepharose. At pH 8.0, the *im*-Al^{3+}− and *im*-Yb^{3+}−OPS bound LYS less effectively than CM-Sepharose. With the exception of *im*-Al^{3+}−OPS, MYO bound to all *im*-M^{n+}−OPS and the *im*-metal ion free OPS and CM-Sepharose adsorbents at pH 5.5, whereas no binding occurred at pH 8.0. At pH 5.5, MYO bound to CM-Sepharose more strongly than to the *im*-M^{n+}−OPS. The metal ion free OPS bound all three proteins at pH 5.5 and 8.0 with affinity similar to that of *im*-Ca^{2+}−OPS, thus exhibiting a different protein selectivity compared to the other *im*-M^{n+}−OPS.

(C) Investigations with Immobilized 8-HQ Adsorbents. As is evident from Table 3, CYT bound to all *im*-M^{n+}−8-HQ chelates at pH 5.5 and was not eluted from the *im*-Al^{3+}−, *im*-Fe^{3+}−, and *im*-Yb^{3+}−8-HQ with 0.3 M NaCl at this pH value. *im*-Cu^{2+}− and Ca^{2+}−8-HQ or the metal ion free 8-HQ and CM-Sepharose all bound CYT, requiring 0.3 M NaCl to elute this protein. At pH 5.5, *im*-Cu^{2+}− and *im*-Ca^{2+}−8-HQ or the metal ion free 8-HQ, however, bound CYT much more strongly than CM-Sepharose. At pH 8.0, CYT bound to all of the *im*-M^{n+}−8-HQ, metal ion free 8-HQ, and CM-Sepharose adsorbents. Interestingly, at pH 8.0, *im*-Yb^{3+}−8-HQ bound CYT most strongly of all the *im*-M^{n+}−8-HQ, with CYT not desorbed with an elution buffer containing 0.3 M NaCl. This result represented the only example of an *im*-Yb^{3+}−chelate with an affinity for any of the test proteins greater than that for the other *im*-M^{n+}−chelates. The metal ion free *im*-8-HQ bound CYT with much lower relative affinity, compared to the *im*-M^{n+}−8HQ, while CM-Sepharose bound CYT most weakly at pH 8.0. LYS also bound strongly to all the *im*-M^{n+}−8-HQ and *im*-metal ion free 8-HQ and was not eluted by 0.1 and 0.3 M NaCl at pH 5.5 or 8.0. In contrast, LYS was partially eluted from CM-Sepharose at pH 5.5 with 0.3 M NaCl and completely eluted with 0.3 M NaCl at pH 8.0. Similarly, MYO bound to all *im*-M^{n+}−8-HQ and the *im*-metal ion free 8-HQ at pH 5.5 but did not bind at pH 8.0 to any adsorbent. At pH 5.5, MYO was not eluted from the immobilized Al^{3+}−, Cu^{2+}−, or Fe^{3+}−8-HQ adsorbent with 0.3 M NaCl and remained bound to *im*-Ca^{2+}− and *im*-Yb^{3+}−8-HQ and metal ion free *im*-8-HQ under these experimental conditions. CM-Sepharose bound MYO more weakly than any of the *im*-M^{n+}−8-HQ or the *im*-metal ion free 8-HQ adsorbents.

Protein Binding Behavior of the Immobilized M^{n+}−Chelate as a Function of Immobilized Chelating Agents at Different pH Values

The comparative influences of the different chelating ligands are summarized in Table 3. Firstly, at pH 5.5, the test proteins (with the exception of the three examples noted below) bound to the *im*-M^{n+}−8-HQ more strongly than to the *im*-M^{n+}−IDA, which in turn bound these proteins more strongly than the *im*-M^{n+}−OPS adsorbents. A similar trend was also evident for the *im*-metal ion free adsorbents. An exception to the above trend occurred with the *im*-Cu^{2+}−OPS and *im*-Cu^{2+}−IDA, where all proteins bound to the former adsorbent much more strongly. Secondly, the three proteins bound to the *im*-Fe^{3+}−8-HQ with a relative affinity similar to that evident with the *im*-Fe^{3+}−IDA. Thirdly, the *im*-Yb^{3+}−IDA and *im*-Yb^{3+}−OPS bound the three proteins with similar apparent affinity. Since the ligand densities of the *im*-M^{n+}−8-HQ and the *im*-M^{n+}−OPS were similar (e.g. 3.3×10^{-4} mol of 8-HQ/g of dry Sepharose CL-4B compared to 4.0×10^{-4} mol of OPS/g of dry Sepharose CL-4B, respectively) but less than the ligand density of the *im*-M^{n+}−IDA (6.0×10^{-4} mol of IDA/g of dry Sepharose CL-4B), these differences in relative binding behavior cannot be ascribed to ligand capacity dependencies or ligand accessibilities. Moreover, at pH 8.0, different trends in protein adsorption behavior for these immobilized M^{n+}−chelate adsorbents were evident. Although the *im*-M^{n+}−8-

HQ still bound the three test proteins with the greatest relative affinity at pH 8.0, under these higher pH conditions, the *im*-M^{n+}−OPS for most M^{n+} ions bound these proteins more strongly than the *im*-M^{n+}−IDA. The binding behavior of the *im*-Fe^{3+}−chelates with LYS and the *im*-Al^{3+}− and Ca^{2+}−chelatets with CYT were noticeable exceptions to this trend.

At pH 5.5, the metal ion free adsorbents generally exhibited the following order of chelate selectivity for the test proteins: 8-HQ > IDA > OPS. Such an order differs from the trend noted for the *im*-M^{n+}−chelates of Cu^{2+}, Fe^{3+}, and Yb^{3+}, while it is similar for chelated Al^{3+} and Ca^{2+}. At pH 8.0, the relative affinity order exhibited by the metal ion free adsorbents was 8-HQ > OPS = IDA. Given that the metal ion content of the *im*-M^{n+}−chelates at pH 5.5 and 8.0 was similar, the trends noted in relative affinity at these pH values with the *im*-M^{n+}−chelates cannot be attributed to adventitious protein interaction with any metal ion free sites which may be present on the *im*-M^{n+}−chelates.

DISCUSSION

With conventional types of IMAC adsorbents involving borderline metal ions, the binding of proteins to the *im*-M^{n+}−chelate complex is usually carried out at relatively high ionic strength (e.g. 0.5−1.0 M) to suppress ionic interactions between the protein and the chelating complex and to promote the paired electron type of coordination bonding (Ramadan & Porath, 1985; Sulkowski, 1988; Kagedal, 1989; Porath, 1990; Arnold, 1991; Wong et al., 1991; Wirth et al., 1993) between the metal ion, such as Cu^{2+} or Ni^{2+}, the chelating ligand, and the protein. Despite the widespread use of borderline Lewis metal ions in the IMAC of proteins, much less attention has been given to the use of immobilized hard Lewis metal ions or the contributing effect of the immobilized chelating agent itself on the separation of proteins with low-ionic strength buffers. One consequence of employing buffers of lower ionic strength is the opportunity to exploit new selectivity options in IMAC, particularly with hard metal ions that are capable of undergoing hydrolytic coordination. The experimental results arising from the present study document that such immobilized hard metal ion chelate complexes, when used with low-ionic strength buffers, can exhibit selectivity behavior typical of pseudocation exchangers.

The surface distribution of charge groups of a protein dominates the binding characteristics (Kopaciewisz et al., 1983; Aguilar & Hearn, 1991) in conventional ion exchange chromatography. For the present investigations, the choice of the three proteins employed was based on their surface charge distribution and their p*I* characteristics. These properties were reflected in their adsorption behavior with the conventional cation exchanger, CM-Sepharose, which was used in these experiments as a control adsorbent. As documented in the Results, the 8-HQ-, OPS-, or IDA-based *im*-M^{n+}−chelate adsorbents, irrespective of the M^{n+} ion used or the chemical nature of the chelating agent, also exhibited selectivity behavior with low-ionic strength loading buffers typical of a cation exchanger. For example, all of the *im*-M^{n+}−chelates with the exception of *im*-Al^{3+}−OPS and *im*-Yb^{3+}−IDA bound MYO at pH 5.5 but not at pH 8.0. Similarly, all of the *im*-M^{n+}−chelates bound LYS and CYT, with the exception of *im*-Cu^{2+}−IDA at pH 5.5 and 8.0. With the exception of the interaction of CYT or LYS with *im*-Fe^{3+}−IDA and most of the *im*-M^{n+}−8-HQ complexes where very strong binding was observed, the bound proteins could be eluted from the other *im*-M^{n+}−chelates in a manner analogous to cation exchange behavior with an increasing NaCl concentration in the elution buffer up to 0.3 M.

The major significant difference in the binding behavior between the conventional cation exchanger, CM-Sepharose, and the *im*-M^{n+}−IDA and *im*-M^{n+}−OPS was, however, evident from the experimental data on the effects of pH. For example, the IDA- and OPS-based adsorbents bound the three test proteins more strongly at pH 8.0 than at pH 5.5. Since all three proteins would be more negatively charged at the higher pH value, they would be expected to progressively bind more weakly to a conventional cation exchanger under these conditions. The stronger interaction of these proteins to the *im*-M^{n+}−chelates at pH 8.0 compared with that of pH 5.5 appears to have its origin in the significantly increased negative charge characteristics of the *im*-metal ion−chelate complex, as the pH becomes higher due to the formation of metal ion−hydroxide ion complexes, rather than the metal ion−hydrated (aquo) complexes. In particular, Fe^{3+} ions are known (Baes & Mesmer, 1976) to form in aqueous solution hydrolytic species at pH values as low as pH 1.5. It can, in addition, be noted that the metal ions used in these studies are also capable of forming complexes with other anions such as chloride and carbonate as well as hydroxide ions in solution. In the present studies, a low concentration of chloride ions was introduced into the adsorption buffer B during the pH adjustment, but this low concentration and the low affinity of chloride ions for the immobilized metal ions compared to hydroxide ions was not expected to lead to very high numbers of *im*-M^{n+}−chelate/chloride ion complexes being formed. Similarly, carbonate complexes are known to form with metal ions, particularly, Ca^{2+} ions, but the participation in the protein adsorption of *im*-M^{n+}−chelate/carbonate ion complexes arising from exposure of the buffer solutions and adsorbents to air was considered to be unlikely. Similarly, in this context, because the ionized Mes an Hepes buffers are known to exhibit poor metal ion binding properties (Good et al., 1966), the formation of metal ion coordination complexes between these species and the immobilized metal ions was considered unlikely.

On the basis of preliminary findings, Ramadan and Porath (1985) proposed an empirical expression for the net charge on *im*-Fe^{3+} ions when present in a chelating adsorbent. For the purposes of the present investigations, modification of this earlier expression was required (Zachariou & Hearn, 1995) in order to accommodate the more generalized case of the different *im*-M^{n+}−chelate complexes used in these IMAC experiments. Evaluation of the overall net charge present on the different *im*-M^{n+}−chelates when the formation of hydrolytic complexes occurs can be achieved from the derived relationship given in eq 1:

$$Q_N = [L(n,q)M(H_2O)_m(OH^-)_p]^{+z-(p+q)} \qquad (1)$$

where Q_N is the overall net charge, L is the immobilized chelating agent with n dentation sites and a charge of q, M is the chelated metal ion with a charge of $+z$, and the term $n + m + p$ is equal to the coordination number of the metal ion. When $p + q$ is greater than $+z$, then the *im*-M^{n+}−

Table 4: Charge−Coordination Dependencies of the Different Immobilized M^{n+}−Chelate Complexes[a]

metal ion	hydrolytic complex at pH 5.5	net charge of complex			hydrolytic complex at pH 8.0	net charge of complex		
		IDA	OPS	8-HQ		IDA	OPS	8-HQ
Al^{3+}	$[L\cdot Al(OH)_2^+]$	−1	−1	0	$[L\cdot Al(OH)_4^-]$	NA	NA	−2
	$[L\cdot Al^{3+}$	+1	+1	−2	$[L\cdot Al(OH)_3]$	−2	−3	−1
	$[L\cdot Al(OH)_3]$	−2	−2	−1				
	$[L\cdot Al(OH)_4^-]$	NA	NA	−2				
Ca^{2+}	$[L\cdot Ca^{2+}]$	0	0	+1	$[L\cdot Ca^{2+}]$	0	−1	+1
Cu^{2+}	$[L\cdot Cu^{2+}]$	0	0	+1	$[L\cdot Cu(OH)^+]$	−1	−2	0
					$[L\cdot Cu(OH)_2]$	NA	NA	−1
					$[L\cdot Cu^{2+}]$	0	−1	0
Fe^{3+}	$[L\cdot Fe(OH)_2^+]$	−1	−1	0	$[L\cdot Fe(OH)_3]$	−2	−3	−1
	$[L\cdot Fe(OH)_3]$	−2	−2	−1	$[L\cdot Fe(OH)_4^-]$	NA	NA	−2
Yb^{3+}	$[L\cdot Yb^{3+}]$	+1	+1	+2	$[L\cdot Yb(OH)^{2+}]$	0	−1	+1
					$[L\cdot Yb(OH)_2^+]$	−1	−2	0
					$[L\cdot Yb(OH)_3]$	−2	−3	−1
					$[L\cdot Yb(OH)_4^-]$	−3[b]	−4[b]	−3
metal free L	NA	−1	−1	0	NA	−1	−2	0

[a] L = immobilized chelating ligand. NA = not applicable. The above hydrolytic species were determined from the following equilibrium constants: Fe^{3+}, $K_1 = 2.7$, $K_2 = 3.8$, $K_3 = 6.6$, and $K_4 = 9.3$; Al^{3+}, $K_2 = 5.8$, $K_3 = 6.0$, and $K_4 = 6.2$; Yb^{3+}, $K_1 = 7.7$, $K_2 = 8.05$, $K_3 = 8.35$, and $K_4 = 10.5$; Cu^{2+}, $K_1 = 8.0$, $K_2 = 9.3$, and $K_3 = 10.5$; and Ca^{2+}, $K_1 = 12.7$. It is assumed that Cu^{2+} has four coordination sites, while all the others are assumed to have six. [b] Ytterbium can have more than six coordination sites, and therefore, it is feasible that several species to exist. It is also assumed that the immobilized chelating agents have tridentate dentation, except for OPS at pH 5.5, where OPS may have bidentate and tridentate geometries.

chelate complex will carry a net negative charge; i.e. it will exhibit a charge characteristic similar to that of a cation exchanger. When $p + q$ is less than $+z$, then the *im*-M^{n+}−chelate complex will carry a net positive charge; i.e. it will exhibit a charge characteristic similar to that of an anion exchanger. When $p + q = +z$, then the net charge on the *im*-M^{n+}−chelate complex will be 0 and no ion exchange behavior is expected. In general, the formation of hydrolytic species of the type $M^{n+}...(OH^-)_p$ rather than the hydrated species $M^{n+}...(OH_2)_m$ will be more favored at higher pH values.

Free OPS has four titratable groups (Eger et al., 1968), with $pK_{al} < 2.0$, $pK_{a2} = 2.1$, $pK_{a3} = 5.7$, and $pK_{a4} = 9.6$. The similarity of the pK_a values of monomeric OPS in solution and *im*-OPS−Sepharose CL-4B has been confirmed in independent investigations (M. Zachariou and M. t. W. Hearn, manuscript in preparation, 1995). Moreover, other immobilized monomeric chelating agents have been shown to have similar pK_a values, as found with their solution counterparts (Szabadka). It is known (Zachariou et al., 1993) that OPS can act as either a bidentate or tridentate chelating agent. For a hexacoordinate M^{3+} ion bound in a bidentate manner through the carboxyl and amine group of *im*-OPS, from eq 1, $n = 2$, $q = -2$, and $m + p = 4$. When p is greater than 1, then the *im*-M^{3+}−OPS complex will exhibit a net negative charge. When p is 0 or 1, then the complex will exhibit a positive or zero charge, respectively. On the other hand, bidentate chelation of *im*-OPS to a quatrocoordinate M^{2+} ion such as Cu^{2+}, via the OPS carboxyl and amine groups, will result in $0 < p < 2$. When $p > 1$, the quatrocoordinate complex will exhibit a net negative charge. In the case of the *im*-OPS binding to a hexacoordinate M^{3+} ion in a tridentate manner, the *im*-M^{3+}−OPS, by including the phosphate group as part of the M^{3+} ion chelation, will have $n = 3$, $q = -3$, and $m + p = 3$. When p is 1 or greater, then a hexacoordinate *im*-M^{3+}−OPS complex will be negatively charged, while when p is 0, the complex will be neutral. In the case of *im*-OPS binding to the quatrocoordinate Cu^{2+} ion in a tridentate manner, even if there are no hydroxyl ions coordinated to the chelated Cu^{2+} ion, the overall net charge will be −1. As the pH becomes higher and the coordinated water is displaced in hydroxyl ions, the net charge on the immobilized quatrocoordinate Cu^{2+}−OPS complex will increase to −2.

Table 4 lists the charge−coordination dependecies of these metal ion chelate complexes. It is apparent that the charge and dentation of the immobilized chelating agent, the charge and coordination number of the metal ion, as well as the ability of the metal ion to intercalate with hydroxyl ions will all contribute to the overall charge on the immobilized M^{n+}−chelate complex. Importantly, the coordination numbers of the *im*-M^{n+}−chelate will depend on the nature of the M^{n+} ion. Yb^{3+} and Ca^{2+}, for example, are capable of having coordination numbers greater than 6 and up to 10, while Fe^{3+} and Al^{3+} will usually be hexacoordinate. Interconversions between the coordination geometry of the multidentate M^{n+}−chelate complex at pH 5.5 and the M^{n+}−chelate complex at pH 8.0, involving hydroxide ions of lower coordination geometry, would be consistent with the transitions to more cation exchange-like properties found in the present investigations with these *im*-M^{n+}−chelates. The participation of these dual properties, involving the interplay of coordination bonding and electrostatic effects, would also explain the relative changes in protein selectivity observed with the *im*-M^{n+}−OPS and metal ion free *im*-OPS at pH 8.0 when compared to that of CM-Sepharose. Previous observations by Sulkowski (1988) on the interaction of phosphoproteins with an *im*-Fe^{3+}−monohydroxamate adsorbent would also be consistent with the involvement of hydrolytic complexes of immobilized hard metal ions in the adsorption process.

Similar considerations can be applied to the tridentate IDA complex ($pK_{a1} = 1.8$, $pK_{a2} = 2.5$, and $pK_{a3} = 9.4$) and to the bidentate 8-HQ complex ($pK_{a1} = 5.1$ and $pK_{a2} = 9.4$) which have also been shown (Szabadka, 1982; Zachariou et al., 1993) to have similar pK_a values when immobilized to Sepharose CL-4B. At pH 5.5, *im*-IDA has a net charge of −1, while at pH 8.0, this chelate would still have a net charge of −1. At pH 5.5, *im*-IDA would have a charge similar to that of *im*-OPS, but at pH 8.0, the latter chelating compound would assume a more negative character. This difference

in charge density would explain the higher relative affinities of *im*-M^{n+}−OPS at pH 8.0 despite it having a lower ligand density than the *im*-IDA. *im*-8-HQ also followed the same trend. Since 8-HQ is a bidentate chelating agent, an additional coordination site would be available on the chelated metal ion for coordination with hydroxide ions or H_2O compared to the situation found with *im*-IDA or immobilized OPS. The greater affinity shown by *im*-M^{n+}−8-HQ for proteins, at pH 8.0, may thus be a direct result of the bidenate nature of the 8-HQ moeity, although the contributory effect of the hydrophobic spacer arm onto which 8-HQ is attached may also have a role (Zachariou et al., 1993; Zachariou & Hearn, 1995).

The higher relative affinities of the three test proteins for the *im*-M^{n+}−chelates at pH 8.0, compared to that for CM-Sepharose, can thus be rationalized on the ability of these hard metal ion IMAC adsorbents to form hydroxide ion-bound complexes which results in a significant increase in the negative charge character of the *im*-M^{n+}−chelate complexes. The binding of proteins to soft or borderline metal ion IMAC adsorbents at high ionic strengths has traditionally been attributed to the involvement of surface accessible histidine, tryptophan, or cysteine residues or phosphate groups. CYT has no surface-exposed histidine, tryptophan, cysteine, or phosphate group moieties (Dickerson et al., 1971; Takano et al., 1973), yet this protein binds (Zachariou et al., 1993; Zachariou & Hearn, 1995) under low-pH, high-ionic strength conditions to immobilized hard Lewis metal ions complexed to 8-HQ- and OPS-based adsorbents and appears to do so through clusters of surface accessible aspartic and glutamic acids via coordination type bonding. These amino acid residues will be negatively charged at pH 5.5 and 8.0 and are thus unlikely to be directly involved in the interaction at low ionic strengths with an overall negatively charged *im*-M_n^{+}−chelate complex. However, CYT has 16 lysine residues (Dickerson et al., 1971; Takano et al., 1973), which will all be positively charged at pH 5.5 and 8.0. The involvement of clusters of these basic amino acid residues would appear to make these the likely contributors to the binding of CYT to the *im*-M^{n+}−chelate complexes under low-ionic strength conditions. Involvement of arginine residues in CYT binding to immobilized hard metal ion chelate adsorbents is unlikely since both arginines are located internally (Takano et al., 1973; Kabasch & Sanders, 1983). MYO has 11 histidines, but only 5 are exposed on the protein surface (Kabasch & Sanders, 1983). The inclusion of 30 mM imidazole in the equilibration buffer has been previously shown to be sufficient to prevent this protein from binding through surface-exposed histidine residues to IMAC adsorbents under high-ionic strength conditions (Zachariou & Hearn, 1995). MYO has also been shown to bind to immobilized hard Lewis metal ion or Cu^{2+}−IDA and OPS adsorbents at high ionic strengths through carboxyl group clusters (Zachariou et al., 1993). As with CYT, the presence of such negatively charged clusters would impede the interaction of MYO with negatively charged *im*-M^{n+}−chelate complexes at pH 8.0. The likely structural contributors to the binding of MYO to the *im*-M^{n+}−chelates under these conditions are one or more of the surface-exposed 18 lysines and the single accessible arginine residue. Similarly, LYS has 4 lysines and 11 arginines exposed on its surface, and again the experimental results are consistent with the participation of one or more of these residues in the electrostatic interaction. Although LYS has been shown to bind to *im*-Fe^{3+}−IDA in 0.5 M NaCl at pH 5.5 through clusters of aspartic and glutamic acids (Zachariou et al., 1993; Zachariou & Hearn, 1995), these charged amino acid side chain groups are unlikely to be involved in the interaction with the negatively charged *im*-M^{n+}−chelate complexes at pH 8.0.

These results indicate that significant selectivity differences exist between the different immobilized hard M^{n+}−chelate adsorbents. In particular, Al^{3+}, Fe^{3+}, and Yb^{3+} bind proteins strongly when chelated to *im*-8-HQ, while Fe^{3+}, Al^{3+}, and Cu^{2+} bind proteins when complexed with the immobilized chelates IDA and OPS. Reversals in protein selectivity can also be seen, as is evident in the case of *im*-Al^{3+}−OPS which did not bind MYO, bound CYT only relatively weakly, but bound LYS very strongly at pH 5.5. These selectivity differences are not a result of differing metal ion content in the respective immobilized hard M^{n+}−chelate adsorbents or a result of protein interactions with metal ion free sites of the immobilized chelator. For example, at pH 5.5, CYT could be eluted in a quantitative manner with 0.1 M NaCl from the *im*-Yb^{3+}−IDA, yet this protein was still significantly bound to the metal ion free IDA gel under the same ionic strength conditions. Similarly at pH 5.5, LYS bound to the *im*-Al^{3+}−OPS and required 0.1 M NaCl to be eluted but required 0.3 M NaCl to be eluted from the metal ion free *im*-OPS. Only in the case of the *im*-Ca^{2+}−chelates was a consistently similar elution conditions behavior found with the immobilized metal ion free chelates.

The selectivity differences noted above for the protein−*im*-M^{n+}−chelate interactions also reflect the stereochemistry of the *im*-M^{n+}−chelate complex. For example, Cu^{2+} is more likely to form a square planar type arrangement, whereas Fe^{3+} will form an octahedral type arrangement (Glusker, 1991). Hydrolytic effects with hydroxyl ions replacing water molecules clearly affect (Martin, 1991) the coordination number of several of the metal ions, such as Al^{3+}. This pH dependent behavior of the *im*-M^{n+}−chelates therefore offers additional selectivity options with proteins when adsorption and elution conditions are selected such that these *im*-M^{n+}−chelates can act as psuedocation exchangers. When used in the low-ionic strength, higher-pH range mode, the same immobilized chelate adsorbents can be employed with a variety of different hard M^{n+} ions to expand the selectivity range. Alternatively, different immobilized chelating agents can be used in combination with the same metal ions in a manner analogous to the concept of tandem immobilized metal ion affinity chromatography (Kagedal, 1989). The use of immobilized hard M^{n+}−chelate complexes as cation exchangers moreover offers the additional option of using the metal ion free immobilized chelate adsorbents as cation exchangers in their own right. Since a wide variety of immobilized chelating agents are commercially available but have until now been predominately used to separate cations or small ionizable organic compounds (Bellinger et al., 1973), considerable flexibility in exploitation of new types of chelators (Chaouk & Hearn, 1994) for the isolation of proteins should thus exist. In this study, the potential of immobilized hard M^{n+}−chelate adsorbents to selectively retain proteins in the mixed coordination−electrostatic mode has been examined. These studies have confirmed the feasibility of employing *im*-Fe^{3+} and other hard Lewis M^{n+} ions such as Al^{3+}, Ca^{2+}, and Yb^{3+} with two recently

introduced IMAC chelating ligands in this mixed mode manner which offers the additional flexibility of generating different selectivity options to those hitherto existing for the separation of proteins by alternative IMAC procedures.

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