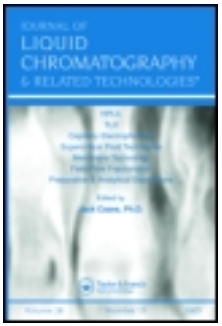

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/ljlc20

Immobilized Iron(III) Metal Affinity Chromatography for the Separation of Phosphorylated Macromolecules: Ligands and Applications

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Version of record first published: 23 Sep 2006.

To cite this article: Leonard D. Holmes & Martin R. Schiller (1997): Immobilized Iron(III) Metal Affinity Chromatography for the Separation of Phosphorylated Macromolecules: Ligands and Applications, Journal of Liquid Chromatography & Related Technologies, 20:1, 123-142

To link to this article: http://dx.doi.org/10.1080/10826079708010641

J. LIQ. CHROM. & REL. TECHNOL., 20(1), 123–142 (1997)

IMMOBILIZED IRON(III) METAL AFFINITY CHROMATOGRAPHY FOR THE SEPARATION OF PHOSPHORYLATED MACROMOLECULES: LIGANDS AND APPLICATIONS

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ABSTRACT

This review highlights the advances in Fe(III)-Immobilized Metal Affinity Chromatography [Fe(III)-IMAC] for the separation and characterization of phosphorylated biomolecules. Fe(III)-IMAC has demonstrated a selective interaction with phosphate groups and other hard Lewis bases such as carboxylate and phenol functional groups. These properties make this technique useful for separating and studying macromolecules. Since phosphorylation is a central means of regulation in eukaryotes and prokaryotes, the development of Fe(III)-IMAC is important. Various iron chelating ligands and their properties are discussed. General features of Fe(III)-IMAC are examined and examples of useful separations are considered.

INTRODUCTION

Immobilized metal affinity chromatography (IMAC) is useful for the separation of biomolecules. The choice of chromatographic support, support activation method, the ligand attached, and immobilized metal ion depends on the properties of the biomolecules to be separated. General IMAC methods and applications have been extensively reviewed.[1-4] Many studies have used divalent metal ions such as Cu(II), Ni(II) and Zn(II) and exploited the affinities for bases such as histidine via metal ion coordination.

Several volumes on general chromatography contain special practical sections describing IMAC protocols.[5-6] Recently, Fe(III)-IMAC has found specific application in the separation of phosphorylated macromolecules and other biological substances.

This review covers the general principles of Fe(III)-IMAC, useful Fe(III) chelating ligands, and the application of Fe(III)-IMAC in adsorption affinity techniques for the separation and study of a variety of phosphorylated biological molecules. The physico-chemical properties of immobilized Fe(III) and its interaction with hard Lewis bases such as phosphate or carboxylate oxygens render this metal ion unique and useful for Immobilized Metal-Affinity Chromatography.

Fe(III)-IMAC: GENERAL PRINCIPLES

First Considerations

Porath and Olin[7] suggested metal-ion "hardness and softness" may be helpful for understanding the mechanisms of Fe(III)-IMAC. At pH's above 7, nickel prefers ligands containing "soft" uncharged sulfur and nitrogen atoms as in cysteine and histidine, while Fe(III) has strong affinity for phosphate, sulfate, carboxylic, and phenolic oxygens. Fe(III) is considered a "hard" Lewis acid, thus it will associate more strongly with "hard" bases such as oxygen.[8] Molecules that have hard bases such as phosphates, carboxylates and phenolic groups have high binding constants. Ligands containing soft bases such as amino and sulfhydryl groups have lower constants. Softer Lewis acids such as divalent metal-ions prefer softer atoms such as nitrogen and sulfur. Although sulfur and nitrogen can be electron donors to Fe(III), these generally form weaker complexes.

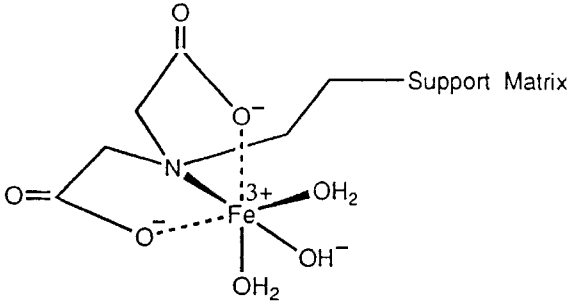

Figure 1. Octahedral Fe(III)-IDA-water complex.

The strength of the coordinate bond between a Fe(III) ion and an immobilized ligand is controlled mainly by three factors:

1. Pure *electrostatic* interaction controlled by the charge and the ionic radius.
2. The overall affinity of the central metal ion to accept electrons from the l ligand.[9]
3. Metal ion electronic configuration which is capable of stabilization due to distortion of octahedral symmetry (Jahn-Teller effect).[9-10]

Metal Coordination or Ion Exchange?

The flexibility of Fe(III)-IMAC stems from the fact that either ion-exchange or ligand coordination properties can be exploited to "tune" specificity. Factors to consider when designing an Fe(III)-IMAC separation are: the net charge of the metal-ligand complex, the ionic strength of the buffer, the presence of surface accessible protein phosphate groups and the isoelectric point of the protein.

Consider the use of immobilized iminodiacetic acid (IDA) as a chelating ligand. Without coordinated metal ion, negatively charged IDA-bonded stationary phases behave as relatively strong cation exchangers.[11] Sulkowski[12] and others[13] showed that a protein mixture could be separated by chromatography over naked bound IDA and that retention increased in order of increasing isoelectric point. When IDA is chelated to Fe(III), the metal ligand complex has a net positive charge (Fig.1).

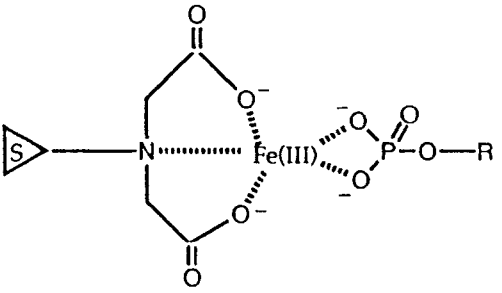

Figure 2. Interaction of Fe(III) and phosphate to form a four-membered ring. (Adapted from reference 19).

This situation is different from that of divalent metal ion-IDA complexes such as Cu(II)-IDA which have no formal charge. For Fe(III) complexes, electrostatic interactions are significant. In this situation, as ionic strength of the buffer is increased, retention of proteins at a pH above their pI (negatively charged) would be expected to decrease due to to shielding of the ligand-metal complex. Proteins of pI 4 to 11 bind to Fe(III)-IDA at pH 6 and low ionic strength.[12]

It has been shown that ion-exchange is the primary binding mechanism for the purification of lactate dehydrogenase by Fe(III)-IMAC.[14,15] It is not displaced from the Fe(III) column by phosphate unless the ionic strength is increased. Ligand-Fe(III) complexes which have no net formal charge bind serum proteins more efficiently than Fe(III)-IDA at low pH, suggesting that nonionic interactions may also play a role in protein-metal complex binding.[7] It may also be useful to consider that steric factors control adsorption or that charge controlled mechanisms occur locally reducing the importance of the net protein charge.

Iron-Phosphate Interactions

The adsorption of phosphate groups to immobilized Fe(III) is not simply the result of electrostatic interactions as negatively charged nucleosides, cyclic mononucleotides and dinucleotides do not bind to IDA-Fe(III),[16] and strength of binding of nucleotides is not affected by the number of phosphates.[17] The high affinity which phosphate has toward Fe(III) is partially explained by the fact that it probably forms two coordinate bonds with the metal ion, whereas, carboxyl groups will form only a single bond (Fig. 2).

The interaction of ferric ion with phosphate has been examined in PEG/dextran two-phase partitioning systems.[18] Affinity for phosphate decreases as alkaline pH is approached. The steep increase in phosphate affinity between pH 3 and 5 is attributed to the ionization of the second acidic oxygen of phosphate. The affinity decreases above pH 5 due to the competitive binding of hydroxyl ions to Fe(III).

Adsorption and Elution

In IMAC, the adsorption of the sample to the resin involves new interactions of the sample with the metal while displacing one or more weakly bound ligands such as water.[3-4,6] When the metal ion binds multiple donor atoms, there is a favorable entropic contribution to the binding energy resulting from displacement of multiple weakly bound ligands (chelate effect).[1,19]

Several methods have been most commonly used for dissociation of phosphorylated molecules from Fe(III)-IMAC columns. They include: pH gradient;[8,20,21] competitive elution with a Fe(III)-binding molecule[22] and elution with Magnesium ion[19] or phosphate ion.[14,23-25]

Mg(II) binds to phosphate groups of the sample, causing desorption. Gradients of NaCl have also been used in Fe(III)-IMAC.[12]

Ovalbumin which contains up to two phosphate groups per molecule, was shown to elute from a Fe(III)-IDA column with a linear gradient of NaCl.[13] This suggested an ionic interaction. However, these experiments were carried out in 25 mM phosphate buffer, explaining the lack of ovalbumin retention. For Fe(III)-IMAC, up to 1 M sodium chloride is often included in the buffer to minimize ion-exchange effects.

NaCl concentrations higher than 1 M do not significantly affect Fe(III)-phosphate interactions.[19] Elution from Fe(III)-IMAC may also be completed by a decreasing salt gradient, because at high salt concentration some proteins may be bound to the stationary phase via hydrophobic interactions.[13]

Samples are easily eluted from iron(III) columns using increasing pH,[13,15,26] or addition of phosphate to the elution buffer to compete off the phosphorylated sample.[27-28] Phosphoserine is also useful as elution agents.[22] Phosphoserine binds to the immobilized Fe(III) displacing the bound sample.

Table 1

Commercially Available Metal-Chelating Supports

Product	Supplier	Support	Ligand	Ref.
Chelating Sepharose Fast Flow	Pharmacia	Cross-linked agarose	IDA	14,19,26
Chelating Sepharose 6B	Sigma	Sepharose 6B	IDA	23
Chelating Amberlite	Sigma	Polystyrene beads	IDA	23
SigmaChrome	Sigma	Poly(hydroxy)-methacrylate	IDA	66
Chelating Superose (a)	Pharmacia	Superose(cross-) linked agarose	IDA	19
Hi-Trap Chelating (a)	Pharmacia	Sepharose, High performance	IDA	19
NTA-Resin	Qiagen	Sepharose-CL-6	NTA	39

(a) Prepacked LC and HPLC columns available.

IRON CHELATING LIGANDS

Almost without exception, commercially available IMAC supports utilize iminodiacetic acid (IDA) as the metal-chelating group. Several available metal chelating supports are listed in Table 1.

Chelating Sepharose 6B® was the first commercially available support to be used for the specific isolation of phosphoproteins by Fe(III)-IMAC.[12,20,29,30] Pre-packed metal affinity columns[31] for fast protein liquid chromatography (FPLC) are also available. These have been used for Fe(III) affinity isolation of phosphopeptides[27] and for model studies of the interactions of Fe(III) ions with phosphorylated amino acids and phosphoproteins.[19]

Table 2

Iron (III) Binding Ligands

Ligand	Coordination	Stability (log K)	Ref.
IDA	3	10.7	7,12,19,37
8-HQ	2	13	37,43,55
Glycylhydroxamate	3	11	37,45,47-48
OPS	3	13	37,50,54
Catechol	2	20	37,67
TED	5	20	7,37
NTA	4	15	37
EDTA	6	25	37
HPO_4^{-2}	2	8.3	37

Numerous volumes are available describing the chemistries and detailed protocols to attach Fe(III)-binding ligands to a natural, synthetic or inorganic chromatographic support.[6,32-34] Fe(III) chelators have been reviewed,[35-36] and only the most common will be discussed. Table 2 shows that Fe(III) prefers binding oxygen and that higher saturation of the six coordination sites forms the most stable complexes. Useful molecules known to chelate iron, the coordination number and their binding constants are tabulated.

Iminodiacetic Acid and its Derivatives

Iminodiacetic acid (IDA) is a "tridentate" iron chelator, with metal coordination through the nitrogen and the two carboxylate oxygen unpaired electrons. Importantly, the metal-ligand complex has 3 coordination sites occupied by weakly bound water which can be displaced by electron rich sites on the molecules to be separated. The iron-IDA complex has a binding constant of log K =10.7 in solution.[37] The iron complex with IDA and water is shown in Fig.1.

Tris(carboxymethyl)ethylendiamine (TED)

Similar to IDA, TED forms a very stable five-membered ring system with Fe(III) (Fig. 3a). The stoichiometry of the metal-chelate complex is not completely defined.[38]

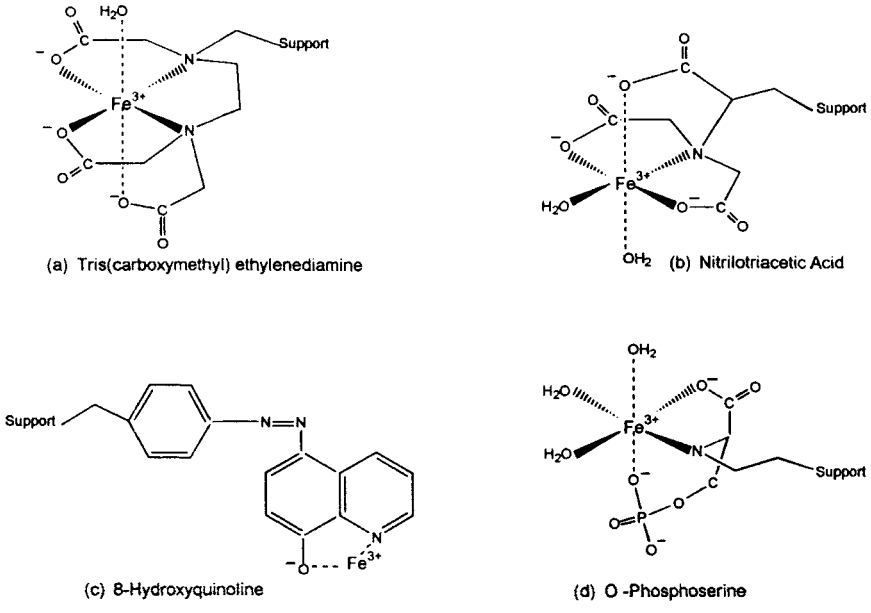

Figure 3. (a) Penta-dentate tris(carboxymethyl)ethylenediamine (TED) complex with Fe(III);(b) Fe(III) complexed to nitrilotriacetic acid (NTA)derivatized support; (c) Immobilized 8-hydroxyquinoline complexed with Fe(III); (d) Fe(III) complexed to o-phosphoserine (OPS) derivatized support.

Nitrilotriacetic Acid (NTA)

NTA contains three carboxymethyl groups attached to a tertiary amine. Four coordination sites of the Fe(III) ion are occupied by the carboxylate oxygens and an amino group. The remaining two coordination positions are occupied by water or hydroxyl ion, depending upon pH. The water and hydroxyl sites are labile and can be displaced by ligands and thus are useful for protein interactions (Fig. 3b).

Ni(II)-NTA agarose has become available from QIAGEN Inc. (39). Preliminary experiments have shown that NTA-Fe(III) is useful for separation of phosphorylated ovalbumin (Unpublished data, Fig. 4).

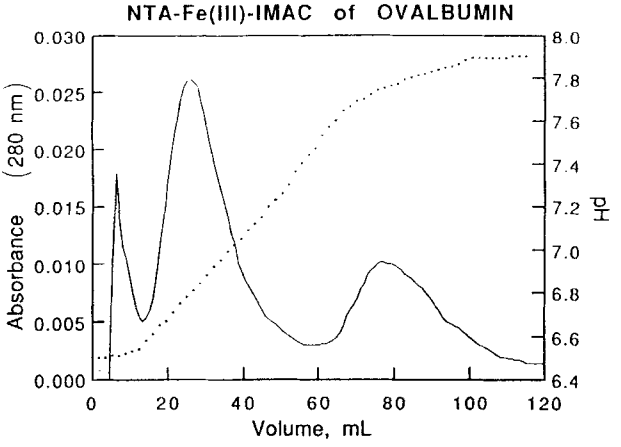

Figure 4. NTA-Fe(III) IMAC of ovalbumin (unpublished result, K.R. Neville and L.D. Holmes).

8-Hydroxyquinoline

8-Hydroxyquinoline (8-HQ) immobilized to silica has long been used for chelation chromatography of transition metal cations.[40-44] The complex of iron and 8-HQ is shown in Fig. 3c.

Hydroxamic Acids

The synthesis and general properties of hydroxamic acids have been extensively reviewed.[36,45-49] They exist as keto-enol tautomeric forms. The keto form is favored at acid pH, and spectroscopic studies indicate that metal complexes are formed with this tautomeric form[48] (Fig. 5). The hydroxamate group is a bidentate chelator that forms a stable 5-membered ring with Fe(III) by loss in free energy during ring formation.

O-Phosphoserine

O-Phosphoserine (OPS) chelation to Fe(III) has been studied by potentiometric titration,[50] and is a useful ligand for Fe(III)-IMAC with an apparent stability constant greater than 10^{13}. The probable structures for the Fe(III)-complex was found to be either ML at low pH and ML_2 at high pH.

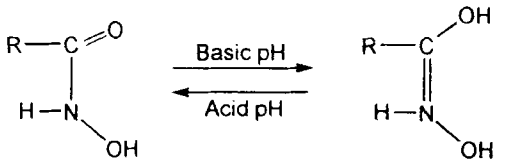

Figure 5. Keto-enol tautomers of a hydroxamic acid.

The chemistries of metal-phosphoserine interactions relating to the role of phosphate esters (e.g. phospholipids) in biochemical processes have been reported.[51-53] The use of OPS-Fe(III) IMAC was recently reported by Zachariou.[54] OPS metal binding is pH-dependent and involves the oxygens in the carboxyl and phosphate groups and the amino nitrogen (Fig. 3d).

EXAMPLES OF Fe(III)-IMAC APPLICATIONS

The usefulness of Fe(III)-IMAC for separation and study of several classes of biomolecules is summarized below.

Amino Acids

Amino acids are zwitter ions and will ionize according to pH. It is interesting to note the contribution nonphosphorylated amino acid residues may make toward the total protein retention in an Fe(III)-IMAC experiment. Cysteine is retained markedly longer on Fe(III)-hydroxymate than any other amino acid.[46] This behavior may be explained by the Lewis base behavior of the sulfhydral group which is chemically similar to hydroxyl and would thus be expected to have appreciable Fe(III) affinity. Cysteine was reported to "destroy" the support in Fe(III)-IDA IMAC due to irreversible binding at low pH.[29] One would anticipate aspartic and glutamic acid residues to show significant Fe(III) interaction through coulombic interactions. Hydrophobic or aromatic amino acids exhibit no special affinity for Fe(III), but phosphoamino acids are strongly retained.[29,46]

Cu(II)-IDA retains amino acids significantly at pH 5 to 6.[13] This is especially interesting for the case of histidine, which strongly interacts with Cu(II).[1,4] Copolymers of glutamate and tyrosine have an affinity for Fe(III)-IDA through carboxylate and phenolic functional groups, although this interaction is weak when compared to phosphate.[16,19] Binding is probably a combination of metal coordination and ion-exchange interactions.

The binding of phosphoamino acids to Fe(III)-IDA Sepharose is very strong compared to that of all amino acids including cysteine, L-aspartic and L-glutamic acids (sulfhydryl and carboxylic group-containing residues). In the absence or presence of high salt, phosphorylated serine, tyrosine and threonine are strongly retained on the IDA-Fe(III) gel at acid pH but not retained at neutral or weakly basic pH.[29] Binding studies using Fe(III)-hydroxamate IMAC demonstrated affinity for surface accessible cysteine, aspartyl and glutamyl residues of proteins.[45-47] Furthermore, binding of proteins to a Fe(III)-8-HQ IMAC resin was demonstrated to be independent of surface accessible histidine, tryptophan or cysteine residues.[55]

Phosphopeptide Studies

The chromatographic behavior of peptides and phosphorylated peptides on Fe(III)-Separose has been analyzed. Basic peptides pass freely through an IMAC column, and acidic peptides are retarded and elute as a broad peak in the pH range 5.5-6.2. Phosphopeptides eluted in the pH range 6.9-7.5.[19]

Certain biological functions of phosphorylation, protein turnover and sequence specificity of protein kinases have been studied by Fe(III)-IMAC of phosphoprotein fragments. Tryptic digests of Photosystem II purified from spinach ^{32}P-labelled chloroplasts were shown to contain phosphothreonine by their retention on Fe(III) chelating Sepharose columns.[20]

Phosphopeptide-mediated intestinal absorption of calcium has been examined Fe(III)-IMAC. Several low abundance caseinophosphopeptides of the pig small intestine have been separated using iron IMAC.[30] Phosphorylated casein proteins are suspected to function as iron transporters and/or storage proteins by forming soluble complexes.[56-59] The physiology of gastric emptying has been studied using Fe(III)-IMAC. Scanff et al.[27] separated low abundance phosphopeptides from a tryptic digest of casein and gastric effluents using an FPLC Fe(III)-Superose HR 10/2 column. Elution was carried out with phosphate buffer.

Secondary, electrostatic interactions were significant, as other non-phosphorylated, strongly basic peptides were also adsorbed to the affinity column. Phosphorylated peptides have been adsorbed with high specificity to Fe(III) immobilized to Whatman paper designed as a counter-part to derivatized Sepharose.[17]

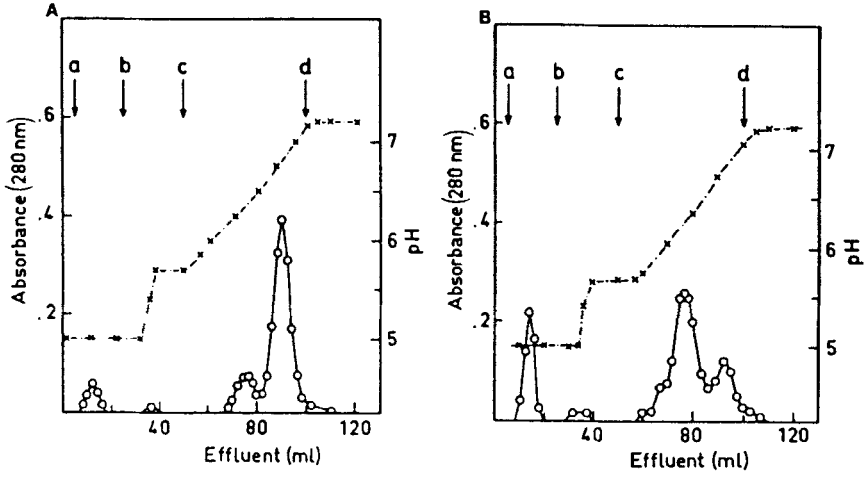

Figure 6. Fe(III)-IMAC of ovalbumin by elution with a pH gradient. (A) Native ovalbumin; (B) partially dephosphorylated ovalbumin. (Used with permission from Ref. 29.)

Phosphoprotein

The first indication that specific phosphate-Fe(III) interactions may be useful for protein separations grew from the work by Porath and Olin comparing the affinities of serum proteins on Fe(III) and Ni(II) columns.[7] The next step was to analyze the Fe(III) binding charateristics of native and dephosphorylated ovalbumin.[29]

At low pH (3.1 and 5.0) the components of native ovalbumin were fractionated according to the number of phosphorylated amino acid residues. Elution of retained proteins was accomplished by pH gradient (Fig. 6).

Similar separation characteristics were found in experiments designed to reduce nonspecific ionic interactions by the use of 1 M NaCl. Enzymatically phosphorylated histone protein showed strong affinity to Fe(III)-IDA.[22] 1 M sodium chloride was used to reduce nonspecific electrostatic interactions. Elution was with a pH gradient, phosphoserine or Mg(II), a phosphate binding ion[59-62] (Fig. 7).

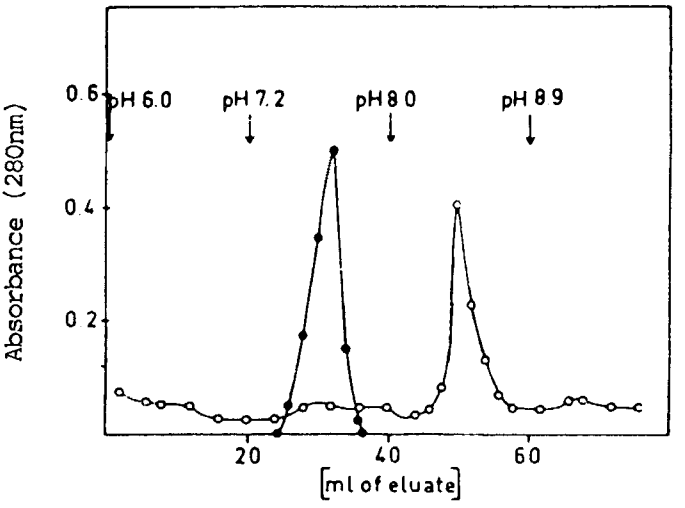

Figure 7. Difference in the pH-dependent elution profiles between phosphorylated (○) and nonphosphorylated histone protein (●) from Fe(III)-IDA gel. (Used with permission from Ref. 22).

Sulkowski[12] studied the affinity of ovalbumin to IDA-Fe(III) and concluded that phosphoproteins display a particularly high affinity for IDA-Fe(III). Secondary interactions involving "nonionic" interactions as well as coulombic interactions were also detected. Ovalbumin has also been purified using metal-affinity partitioning in aqueous two-phase systems.[18] Interestingly, neither TED-Fe(III) or hydroxamate-Fe(III) display any affinity for ovalbumin.[29,46]

Singly phosphorylated pepsin (pI=2.9) was also retained and subsequently eluted with 20 mM phosphate. Furthermore, Kucerova[59] separated human gastric pepsins and pepsinogens by this technique, suggesting that the degree of phosphorylation may be correlated with gastric disease. Michel and Bennett[20] in their studies of the photosynthetic electron transport system, found that phosphoprotein from Photosystem II of spinach was retained on IDA-Fe(III) gel at pH 5.0 and eluted upon increasing the pH to 8.0. Phosphorylation is implicated in photosystem control.[62] Fe(III) immobilized to an agarose-carboxymethyl-picolyamine matrix has been used to isolate insect phospholipoproteins.[28]

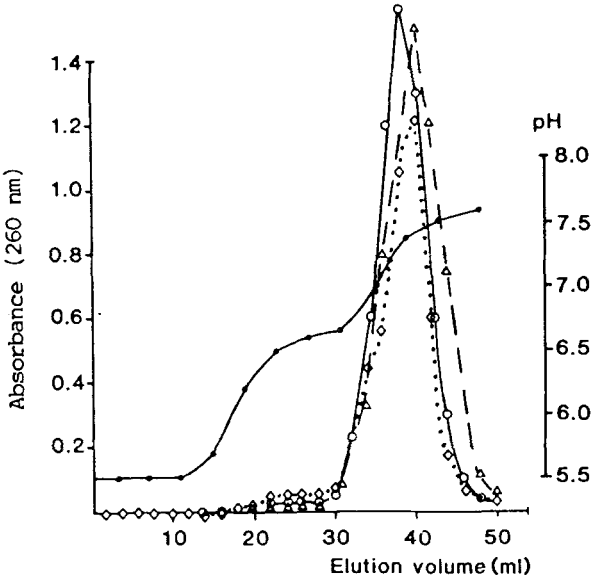

Figure 8. Fe(III)-IMAC elution profiles of adenine nucleotides. AMP (o--o); ADP (Δ--Δ); ATP (◊--◊); pH (●--●). (Used with permission from Ref.16.)

Specific Fe(III)-phosphate interactions are indicated by protein elution with phosphate buffer but not by high salt. Recently, Fe(III)-IMAC was employed for the separation of microtuble-associated proteins (MAPs).[26]

Nucleotides and Nucleic Acids

Nucleosides, cyclic nucleotides, and dinucleotides which contain only internal or no phosphate groups do not bind to an IDA-Fe(III) column at pH 5.5 in the presence of high sodium chloride.[7,12,16] AMP, ADP and ATP co-elute from an Fe(III)-IMAC gel indicating that adsorption of nucleotides is not based entirely on simple electrostatic interactions (Fig. 8).

Binding of nucleotide was found to be through the terminal phosphate when AMP, ADP and ATP were completely retained by IDA-Fe(III) derivatized chromatography paper.[17] Fe(III)- nucleotide phosphate affinity has been used to probe enzyme active site structural features of several nucleotide binding proteins.[24-25,63] Competition binding studies with photoaffinity-labeled azido analoges of NAD^+, ATP, and ADP located peptide sequences at the

adenine binding domains. Also the guanine binding site for glutamate dehydrogenase was identified using this method.[23] Iron(III) IMAC provides a one-step approach for separation of photoaffinity-labeled peptides from non-labeled peptides.

CONCLUSIONS

Fe(III)-IMAC has become a useful technique for the purification and characterization of phosphorylated biomolecules. Phosphorylation modulates enzyme activities in the cell nucleus, mitochondrian, ribosomes and cell membranes. Protein phosphorylation also mediates secretion, molecular transport and membrane permeability.[64-65] The abundance and diversity of phosphorylated molecules and the nature of protein-bound phosphate will drive the development of immobilized iron separation techniques.

Although experiments have reported significant success in achieving phosphate-specific separations, a detailed analysis of the mechanism of adsorption of phosphorylated biomolecules remains to be completed. A wide range of parameters affect chromatographic behavior. Factors such as chelating ligand, support matrix, buffer conditions and temperature require further study in order to optimize analyses. Undoubtedly, a portion of the adsorption behavior is due to general ionic or electrostatic interaction of the metal ion with phosphate or other negatively charged groups. Hydrophobic interactions also must not be ignored. However, Fe(III)-IMAC systems do not interact with phosphoproteins in the same way as ordinary ion exchange resins. Fe(III)-IMAC can be employed to resolve proteins with a wide range of isoelectric points, a task not generally possible in ion exchange chromatography. Fe(III)-IMAC is also effective in probing the accessible surfaces of proteins for the presence of certain amino acids such as aspartate, glutamate and cysteine. This is a useful addition to Cu(II)-IMAC which binds surface histidines.

ACKNOWLEDGEMENTS

LDH is grateful to Professor Frances H. Arnold at the California Institute of Technology (USA) and Professor M.A. Vijayalakshmi at the Universite De Technologie De Compiegne (France) for both financial support and inspiration. Gratitude is expressed to their wonderful research groups for the many hours of instruction and discussion on all aspects of metal affinity chemistry. This work has also been made possible through the generosity of the North Carolina Biotechnology Center.

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Received April 27, 1996
Accepted May 14, 1996
Manuscript 4182