Journal of Chromatography, 604 (1992) 19–28
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 058

Model studies on iron(III) ion affinity chromatography

II.☆ Interaction of immobilized iron(III) ions with phosphorylated amino acids, peptides and proteins

Grażyna Muszyńska☆☆, Grażyna Dobrowolska☆☆, Anders Medin, Pia Ekman and Jerker O. Porath

Biochemical Separation Centre, Uppsala University, Box 577; Department of Biochemistry, Uppsala University, Box 576; and Department of Medical and Physiological Chemistry, Uppsala University, Box 575, S-751 23 Uppsala (Sweden)

ABSTRACT

The chromatographic behaviour of phosphoamino acids, phosphopeptides and phosphoproteins and their non-phosphorylated counterparts was studied on Fe(III)-Chelating Sepharose® and Fe(III)-Chelating Superose®. The phosphorylated compounds, in contrast to their non-phosphorylated or dephosphorylated counterparts, adsorb to immobilized iron(III) ions at pH 5.5 and can be desorbed by an increase in pH. Phosphoamino acids were eluted at pH 6.5–6.7, whereas monophosphopeptides and phosphoprotamine eluted in the pH range 6.9–7.5. Molecules possessing clusters(s) of carboxylic groups are weakly retained (γ-carboxyglutamic acid, Ala-Ser-Glu_5) or bound (polyglutamic acid, β-casein) to the immobilized iron(III) ions at pH 5.5. Dephosphorylated β-casein was desorbed at pH 7.0, whereas for elution of native (non-dephosphorylated) β-casein, phosphate buffer of pH 7.7 was required. The homopolymer of polyglutamic acid was desorbed in the pH range 6.0–6.3, whereas copolymers of glutamic acid and tyrosine require pH 7.0–7.3 or even phosphate buffer at pH 7.7 for elution.

INTRODUCTION

Immobilized (chelated) iron(III) ions interact with phosphate groups of phosphoproteins [1,2]. This interaction has been utilized for the separation of phosphopeptides and phosphoproteins [2–5]. Recent studies on the binding of nucleotides to chelated iron(III) ions have established that a free terminal phosphate group is sufficient for the adsorption [6].

It has also been shown that proteins lacking phosphate groups interact with immobilized iron (III) ions [7,8], suggesting an affinity for other acidic groups in a polypeptide chain [9]. In addition, it has been suggested that exposed aromatic side-chains can promote metal complexation [10,11].

This study was undertaken in order to find out which groups on proteins are involved in the interaction with immobilized iron(III) ions.

EXPERIMENTAL

Materials

Amino acids, polymers of amino acids, proteins, 2-(N-morpholino)aminomethanesulphonic acid (MES), tris(hydroxymethyl)aminomethane (Tris) and iron(III) chloride were purchased from Sigma (St. Louis, MO, USA). The basic peptide,

Correspondence to: Dr. J. O. Porath, Biochemical Separation Centre, Uppsala University, Box 577, S-751 23 Uppsala, Sweden.

☆ For Part I, see ref. 6.

☆☆ Permanent address: Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Rakowiecka 36, 02-532 Warsaw, Poland.

0021-9673/92/$05.00

RRKASGP, was purchased from Bachem (Bubendorf, Switzerland) and the acidic peptide, ASEEEEE, prepared as described previously [12], was a kind gift from Professor F. Marchiori of the Centre of Biopolymers (Padua, Italy). [γ^{32}]ATP was purchased from Amersham International (Amersham, UK). Chelating Sepharose® Fast Flow and Chelating Superose® were purchased from Pharmacia LKB Biotechnology (Uppsala, Sweden). All other chemicals were of reagent grade. The protein kinase A (cyclic AMP-dependent protein kinase) and casein kinase IIB from maize seedlings were prepared as described previously [13–15].

Phosphorylation

Protamine (500 μg) and the basic peptide (500 μg) were phosphorylated separately by protein kinase A (18 600 units) using 10^7 cpm of carrier-free [γ^{32}]-ATP (specific radioactivity 3000 Ci/mmol) as the phosphate donor. The incubation, in a total volume of 200 μl, was performed overnight at 30°C in 20 m*M* MES buffer (pH 6.9) containing 10 m*M* $MgCl_2$. The acidic peptide (400 μg) was phosphorylated by casein kinase (1000 units) using the same amount of radioactive ATP. The incubation, in a total volume of 200 μl, was performed overnight at 30°C in 10 m*M* Tris buffer (pH 7.7) containing 10 m*M* $MgCl_2$. The amount of incorporated phosphate into protamine and the two peptides was detected by determination of the radioactivity by the Cerenkov method.

One phosphorylation unit was defined as the amount of enzyme transferring 1 pmol of phosphate per minute to the protein or peptide acceptor under standard assay conditions.

Protein concentrations

Protein concentrations were monitored by measurements of the absorbance at 280 or 595 nm according to the calorimetric procedure of Bradford [16] using bovine serum albumin as a standard.

Amino acid and peptide concentrations

The concentrations of amino acids and peptides were determined by the ninhydrin reaction at 570 nm [17,18]. The peptides had previously been subjected to alkaline hydrolysis.

Chromatography

The buffers used were as follows: (A) 50 m*M* MES–1 *M* NaCl, pH 5.5; (B) 20 m*M* MES–1 *M* NaCl, pH 6.5; (C) 100 m*M* Tris–1 *M* NaCl, pH 7.5; (D) 20 m*M* sodiumphosphate–1 *M* NaCl, pH 7.7; (E) 50 m*M* Tris–1 *M* NaCl, pH 7.0. All buffers were degassed and filtered prior to use.

Fe(III)immobilized metal ion affinity chromatography (IMAC) on Chelating Sepharose Fast Flow

The degassed gel was packed in columns (15 mm × 10 mm I.D., $V_t \approx 1$ ml) in distilled water and charged with a few column volumes of 20 m*M* iron (III) chloride solution. Excess of Fe(III), not bound or loosely bound, was removed from the columns by washing with 10–15 volumes each of water, buffer C and buffer A. Chromatography was conducted at room temperature with a flow-rate of 15 ml/h; 2.0-ml fractions were collected. In each run 0.4–1.0 mg of substance in 0.5–1.0 ml of buffer A was applied to the column. The columns were washed with 10 ml of buffer A, and then eluted in a sequence with 10 ml of buffer B, a continuous pH gradient formed by the gradual mixing of 17 ml of buffer C with 17 ml of buffer B and finally with 20 ml of buffer D. With β-casein and its dephosphorylated form, 2 mg in buffer B were used and chromatography was started from pH 6.5.

After each experiment the chelating gel was regenerated with 100 m*M* EDTA containing 1 *M* NaCl and then washed with water. The metal-free columns were stored at room temperature and charged with iron(III) ions immediately before use. Every column was recycled several times.

Fe(III) IMAC on Chelating Superose using the fast protein liquid chromatographic (FPLC) system

Chromatography was performed on a Chelating Superose HR 10/2 prepacked column according to the manufacturer's instructions. Loading and stripping of Fe(III) and the conditions for equilibration were the same as for Chelating Sepharose Fast Flow (see above). The same amount of sample was also applied. Programs for three different procedures were developed on the FPLC system for Fe(III) IMAC. The procedures containing directions for charging with the metal, column prewash, sample application, isocratic elution, gradients in pH and $MgCl_2$, stripping of the metal and regeneration of

the column were included in a single program.

Procedure 1. The conditions were as follows: buffer A, 10 ml; buffer B, 20 ml; a continuous Mg(II) gradient from 0 to 1 *M* $MgCl_2$ (70 ml), formed by the gradual mixing of buffer B with buffer B containing 1 *M* $MgCl_2$.

Procedure 2. The conditions were as follows: buffer A, 20 ml; buffer E, 10 ml; a continuous Mg(II) gradient from 0 to 1 *M* $MgCl_2$ (70 ml), formed by the gradual mixing of buffer E with buffer E containing 1 *M* $MgCl_2$.

Procedure 3. The conditions were as follows: buffer A, 30 ml; a continuous pH gradient (30 ml), formed by the gradual mixing of buffer A with buffer C; buffer C, 10 ml; a continuous Mg(II) gradient from 0 to 1 *M* $MgCl_2$ (70 ml), formed by the gradual mixing of buffer C with buffer C containing 1 *M* $MgCl_2$.

RESULTS

Amino acids

None of the tested nonphosphorylated amino acids was adsorbed on the Fe(II)-Sepharose column at pH 5.5. In contrast, phosphoamino acids were bound to the column and desorption was achieved at pH 6.5. Phosphoserine and phosphotyrosine were eluted at pH 6.5 and phosphothreonine at pH 6.75 (Fig. 1).

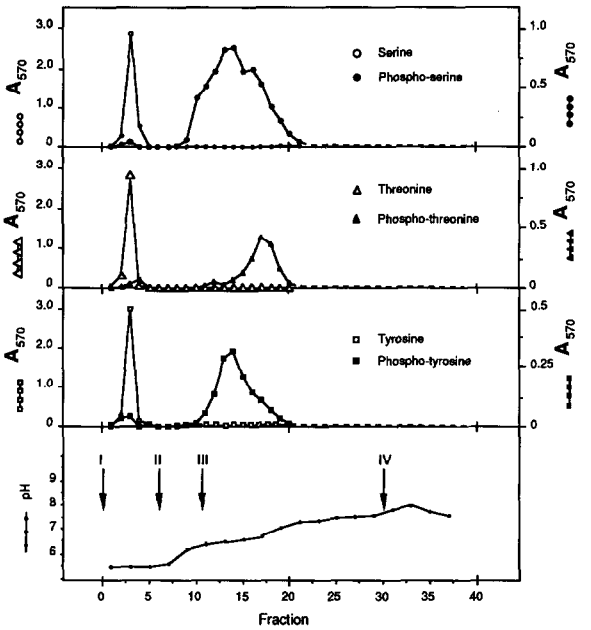

Fig. 1. Elution profiles of phosphoamino acids from Fe(III)-chelated Sepharose. Changes in buffer compositions are marked by arrows: I = buffer A; II = buffer B; III = pH gradient of buffers B and C; IV = buffer D. The internal reference for Tris, which forms coloured complexes with ninhydrin, was always determined along with the amino acid analysis. The absorbance at 570 nm was therefore corrected for the influence of Tris on the absorbance value in the pH gradient.

Peptides

The basic peptide (RRKASGP) behaved like unmodified amino acids and passed freely through the column. In contrast, the acidic peptide (ASEEEEE) was retarded and emerged as a broad peak in the pH range 5.5–6.2. Both basic and acidic peptides, phosphorylated on the single serine residue, were adsorbed. Their desorption was achieved at pH 7.0 and 7.4, respectively (Fig. 2). The difference in pH required for desorption may be attributed to interactions between iron(III) ions and acidic groups, as indicated by the retardation of the peptide containing a cluster of five glutamic acid residues.

A further increase in the number of COOH groups resulted in a slight strengthening of the binding to the gel, as a homopolymer with an average number of 308 glutamic acid residues was eluted in the pH range 6.0–6.3. Some of the material found in the breakthrough peak probably reflects the presence of impurities of the compound of low or even no degree of polymerization.

The random copolymer of poly(Glu, Tyr), having a ratio of glutamic acid to tyrosine of 4:1, was more strongly bound to the gel than the homopolymer of polyGlu, and was eluted as a broad peak in the pH range 6.75–7.45. Total desorption of the polymer was achieved at pH 7.7 in 20 m*M* phosphate buffer. Two other peptide copolymers of similar molecular weight, consisting of Glu–Ala–Tyr and Glu–Lys–Tyr in the proportions 6:3:1, were mainly eluted in relatively sharp peaks with the maximum at pH 7.0 (Fig. 3).

The elution conditions for glutamic acid derivatives are presented in Table I. Glutamic acid and its dipeptide were eluted from the column in the breakthrough fraction; γ-carboxyglutamic acid and a heptapeptide containing five glutamic acid residues were slightly retarded on the column.

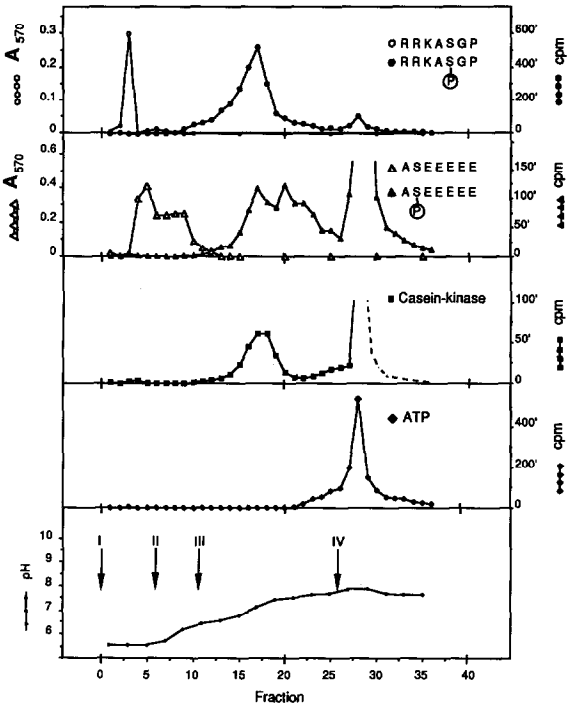

Fig. 2. Elution profiles of phosphopeptides from Fe(III)-chelated Sepharose. Conditions as in Fig. 1. Right-hand scale: primes denote thousands, *i.e.* 600′ is 600 000.

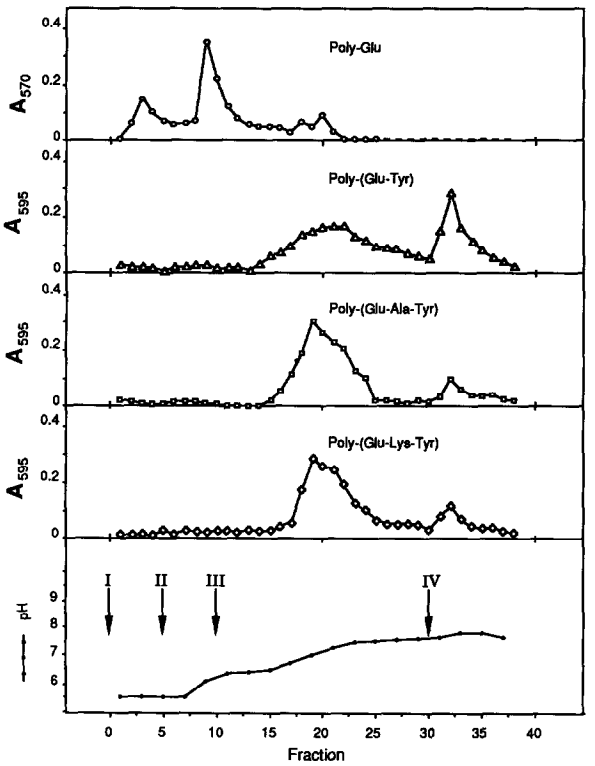

Fig. 3. Elution profiles of polyglutamic acid polymers from Fe(III)-chelated Sepharose. Conditions as in Fig. 1.

Proteins

Owing to the low solubilities of β-casein and its dephosphorylated form at pH 5.5, they were applied on the column at pH 6.5 (Fig. 4a). Native β-casein, having clusters of glutamic acids and a high degree of serine phosphorylation (see refs. 19

TABLE I

CONDITIONS FOR ELUTION OF GLUTAMIC ACID DERIVATIVES FROM IRON(III)-CHELATED SEPHAROSE

Compound	Number of groups		pH of elution
	Carboxylic	Phenolic	
Glu	2	0	5.5
Glu–Glu	3	0	5.5
Ala–Ser–Glu–Glu–Glu–Glu–Glu	6	0	5.5–6.2 (5.5)[a]
γ-CarboxyGlu	3	0	5.5–5.9 (5.5)[a]
PolyGlu	*ca.* 308	0	6.0–6.3 (6.0)[a]
Poly(Glu–Tyr)	*ca.* 210	*ca.* 52	6.7–7.5 (7.2)[a] 7.7[b]
Poly(Glu–Ala–Tyr)	*ca.* 110	*ca.* 18	6.8–7.3 (7.0)[a]
Poly(Glu–Lys–Tyr)	*ca.* 84	*ca.* 14	6.8–7.3 (7.0)[a]

[a] The pH of maximum desorption is given in parentheses.
[b] Elution was achieved by a linear pH gradient, followed by 20 m*M* phosphate buffer.

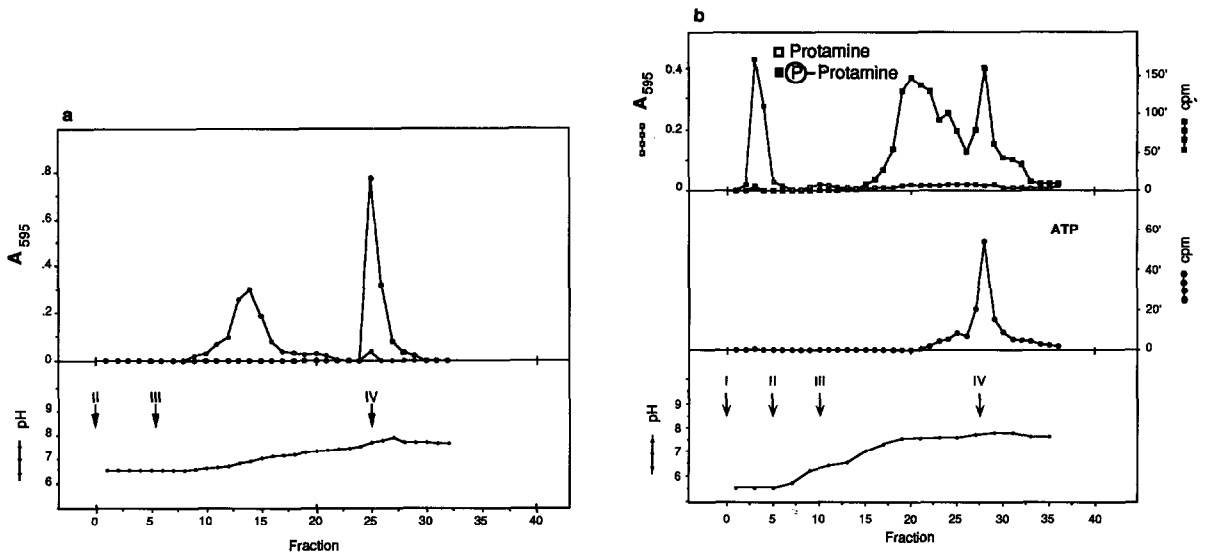

Fig. 4. Elution profiles of phosphoproteins from Fe(III)-chelated Sepharose. (a) ● = β-Casein and ○ = its dephosphorylated form; (b) protamine, phosphorylated and non-phosphorylated. Change of buffers as in Fig. 1.

and 20 and Fig. 5a), was bound very strongly to the immobilized iron(III) ions. For the elution of β-casein, phosphate buffer (pH 7.7) was required. The commercially available, enzymatically dephosphorylated (to about 80%) β-casein did not interact so strongly with the metal. After dephosphorylation the affinity of β-casein for the gel decreased, the major portion of the adsorbed protein was eluted by Tris buffer (pH 7.0) and another minor peak was obtained with phosphate buffer (pH 7.7). As native β-casein required inorganic phosphate to be eluted under these conditions, we conclude that this second peak contains unreacted (native) β-casein (Fig. 4a).

Similarly to other phospho compounds, phosphorylated protamine but not its unmodified form was adsorbed on iron(III)-chelated Sepharose and subsequently eluted at pH 7.5 (Fig. 4b). Three sites on the protamine seem to be available for phosphorylation by protein kinase A (see ref. 21 and Fig. 5b).

Fast protein liquid chromatography (FPLC)

Phosphoprotamine, a basic phosphopeptide and their non-phosphorylated counterparts were chromatographed on iron(III)-chelated Superose columns (prepacked gel bed) with the FPLC system according to procedures 1–3 (see Experimental).

As can be seen in Fig. 6a, in procedure 1, phospho compounds were not eluted at pH 6.5 until the $MgCl_2$ concentration reached 0.4 *M*. All three elution profiles were broad and were composed of several overlapping peaks.

In procedure 2, where the Mg^{2+} gradient started at pH 7.0, phosphorylated protamine started to appear at pH 6.9; four major peaks were eluted at a constant pH of 7.0 with increasing magnesium concentration, up to 0.3 *M*. Phosphopeptide was eluted as a single symmetrical peak at pH 6.9 (Fig. 6b).

In a separate experiment, 3 *M* NaCl instead of the routinely used 1 *M* NaCl was applied. At this sodium chloride concentration the peptide was desorbed at a pH 0.5 unit lower than when the experiment was performed with 1 *M* NaCl (data not shown).

In procedure 3, the increase in pH to 7.5 resulted in the elution of three single, relatively symmetrical peaks, at pH 7.1, 7.3 and 7.5 for basic phosphopeptide, phosphoprotamine and ATP, respectively (Fig. 6c).

The elution conditions for phospho compounds from the iron(III)-chelated gels are summarized in Table II. Basic phosphopeptide was eluted at pH 6.9–7.1 from iron(III)-chelated Sepharose and Superose, whereas for the elution of phosphoprotamine from these gels pH values of 7.5 and 7.3 were required, respectively.

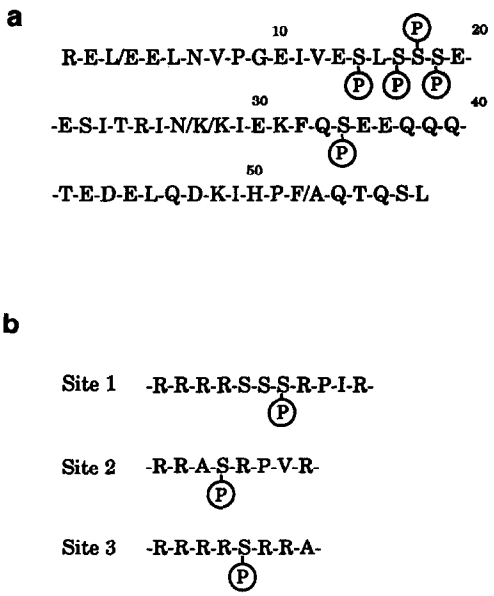

Fig. 5. Phosphorylation sites on (a) β-casein and (b) protamine. (a) Serine groups phosphorylated endogenously by the mammary gland casein kinases that could be removed by alkaline phosphatase are designated by an encircled P. According to Traugh and co-workers [19,20]. (b) Sites on protamine available for phosphorylation by protein kinase A are indicated by an encircled P. According to Bramson *et al.* [21].

DISCUSSION

During the last few years, we have claimed that iron(III) ions, immobilized on chelating gels, display a preferential affinity to phosphate groups in proteins and nucleotides. Our previous results had suggested that an interaction occurs between Fe (III) and the oxygen of the phosphate group [2,6]. The data presented in the first part of this paper have confirmed that suggestion. Unphosphorylated molecules are eluted at a lower pH (pH 5.5) than their corresponding phosphorylated derivatives (Fig. 7).

Although the interactions with immobilized metals have been exploited for phosphopeptide and phosphoprotein separations [2–5], their mechanisms are still not fully clarified.

The present results indicate that the binding of macromolecules to the immobilized iron(III) ions is more complex than assumed earlier [1,2] and is not limited to the iron(III)–phosphate interaction(s). We are now recognizing the role of carboxylic and phenolic groups in the metal ion interaction. Glutamic acid, its derivatives, homo- and heteropolymers (listed in Table I), with different contents of

TABLE II

ELUTION OF PHOSPHOAMINO ACIDS, PHOSPHOPEPTIDES AND PHOSPHOPROTEINS FROM IRON(III)-CHELATED GELS

Compound	Type of chromatography[a]	Elution conditions		Chelating gel
		pH	$[MgCl_2]$ (*M*)	
Phosphoserine	1	6.5	–	Sepharose
Phosphothreonine	1	6.7	–	Sepharose
Phosphotyrosine	1	6.5	–	Sepharose
Ala(Ser P)Glu_5	1	7.4	–	Sepharose
Arg_2Lys–Ala(Ser P)Gly–Pro	1	7.0	–	Sepharose
	2	6.5	0.56–0.90	Superose
	2	6.9	None	Superose
	2	7.1	None	Superose
Phophoprotamine	1	7.5	–	Sepharose
	2	6.5	0.40–0.58	Superose
	2	7.0	0.08–0.30	Superose
	1	7.3	None	Superose
β-Casein (enzymatically dephosphorylated)	1	7.0	–	Sepharose
β-Casein	3	7.7	–	Sepharose

[a] Type of chromatography: 1, linear pH gradient; 2, stepwise increase in pH followed by a linear $MgCl_2$ gradient (0–1 *M*); 3, linear pH gradient followed by 20 m*M* phosphate buffer.

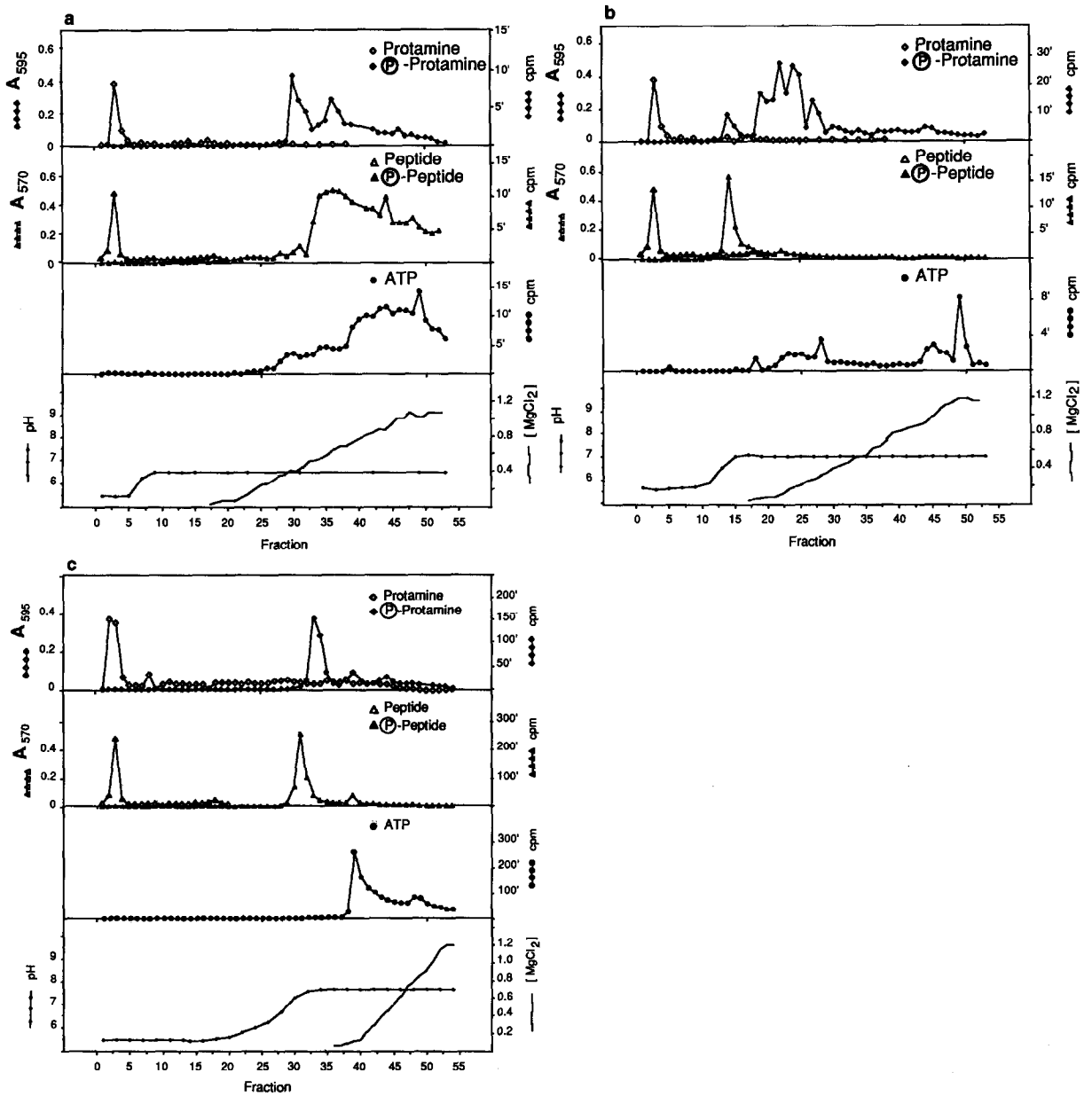

Fig. 6. FPLC separation of phosphopeptide and phosphoprotamine on Fe(III)-chelated Superose. For the three elution conditions, gradient in (a) pH 5.5–6.5, (b) pH 5.5–7.0 and (c) pH 5.5–7.5, procedures I, II and III were used, respectively (for details see Experimental).

glutamic acid and tyrosine residues were chosen for the model studies. Two carboxylic groups in glutamic acid or three in its dipeptide were not sufficient to bind with immobilized iron(III) ions at pH 5.5. However, with three carboxylic groups present very closely assembled, as in γ-carboxyglutamic acid, the elution of the compound from the gel was delayed. A similar chromatographic retardation effect was observed for the acidic heptapeptide consisting of a cluster of five glutamic acid residues.

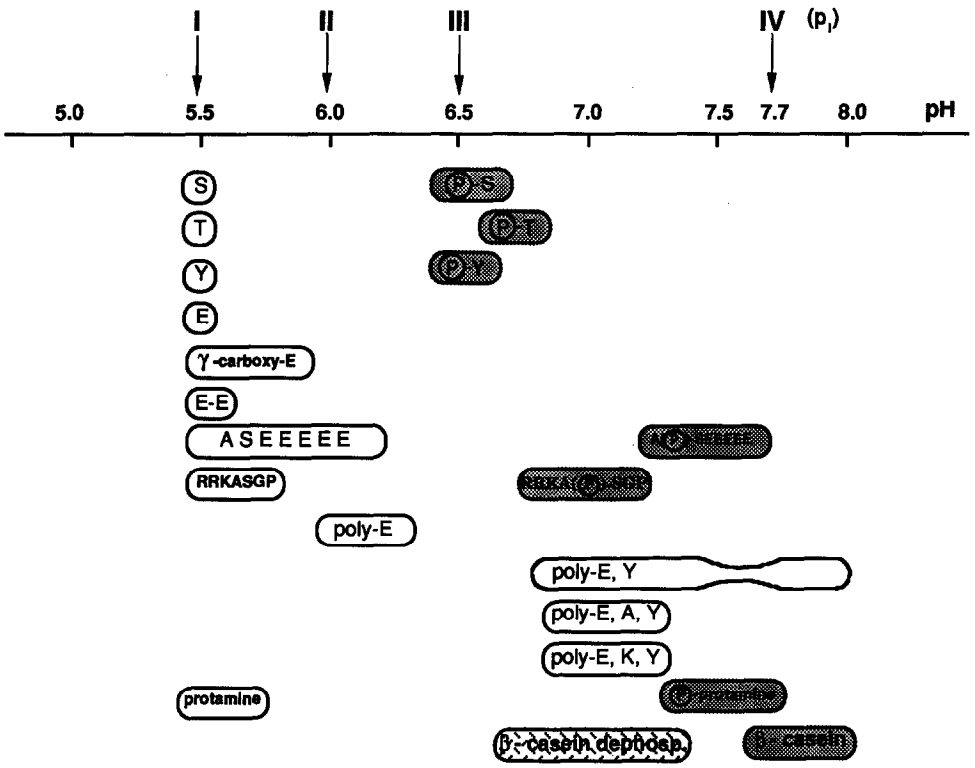

Fig. 7. Comparison of elution, from Fe(III)-chelated Sepharose, of phosphorylated molecules, their non-phosphorylated counterparts and compounds containing different number of carboxylic and phenolic groups. The diagram shows the chromatographic behaviour of amino acids, peptides and proteins. Phosphorylated groups are indicated by an encircled P. Conditions as in Fig. 1; I, II, III, IV = elution buffers A, B, C and D, respectively.

The tremendous increase in carboxylic groups to about 300 on average in the poly-Glu homopolymer results in an increase in the binding strength, but it is still a relatively weak interaction.

It should be pointed out, however, that the copolymers consisting of glutamic acid and tyrosine residues are bound strongly to the iron(III) gels and their desorption requires nearly the same conditions as those for phosphopeptides and phosphoproteins (Fig. 7). The stronger binding of this copolymer than that of the Glu–Ala–Tyr and Glu–Lys–Tyr copolymers may be ascribed to its higher number of phenolic groups (Table I and Fig. 7) and a more direct cooperative effect of adjacent carboxylic and phenolic groups.

The tentative modes of the interaction of phosphate and carboxyl groups with immobilized Fe(III) are presented in Fig. 8. One phosphate group is sufficient for the binding, whereas the second type of interaction is likely to be promoted by the presence of several carboxyl groups. According to recent suggestions [22], phosphate groups could form a four-membered chelating ring, whereas the carboxylic groups might be involved in the formation of a multi-point attachment, probably a six-membered chelating ring. This could account for the stronger binding of phosphate group to the immobilized iron(III) ions compared with that of the cluster of carboxylic groups. The presence of phenolic rings can enhance the binding of adjacent carboxyl groups, presumably by the involvement of π-electrons of the aromatic ring. The lack of experimental data tends to discourage any further speculation at present.

This work demonstrates again that the adsorption of peptides and proteins to chelated iron(III)

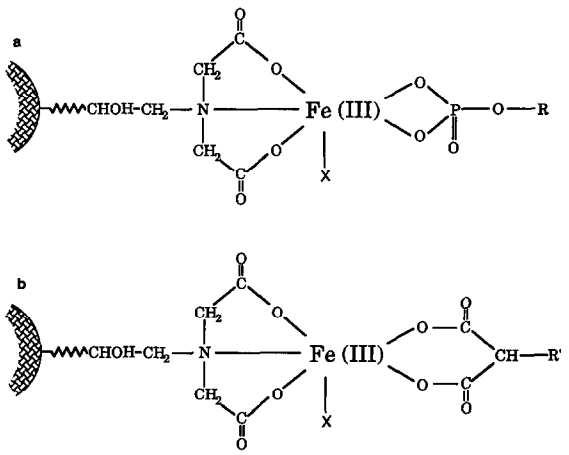

Fig. 8. Postulated mode of interactions of phosphate and carboxyl groups with the immobilized iron(III) ions. (a) Phosphate groups; (b) carboxyl groups. X may be water, hydroxyl, etc., and might also be involved in the binding of macromolecules. R and R' represent the compounds studied.

ions is not based only on a coulombic interaction as the columns were charged in the presence of 1 *M* sodium chloride, precluding simple ion-exchange chromatography. The strength of binding of phosphorylated basic peptide is slightly decreased in the presence of 3 *M* sodium chloride. However, it has previously been observed [2] that the elution of phosphorylated histone from iron(III)-chelated gel was not effected by 4 *M* sodium chloride. The presence of sodium chloride (higher than 1 *M*) does not greatly affect the binding, possibly because this salt is an intermediate on the Hofmeister scale [23].

Magnesium chloride, on the other hand, has a significant effect on the desorption of phospho compounds and is able to desorb these compounds at a relatively low pH (low pH is insufficient in itself for desorption). At low pH, higher concentrations of Mg^{2+} are required for desorption. At pH < 7.0 $MgCl_2$ elutes phospho compounds in a broad peak, without a distinct maximum. At pH 7.0, a linear increase in $MgCl_2$ concentration causes elution of several peaks containing phospho protamine. This may indicate that the separated peaks are protamine with different degrees of phosphorylation. This supports the previous suggestion [2] that, under certain chromatographic conditions, magnesium ions can be useful for the separation of proteins differing in the number of phosphate groups. The desorptive action of Mg^{2+} ions can be interpreted as being due to an interaction between the magnesium ions and the phosphate groups. Apart from magnesium, inorganic phosphate was used as a low-molecular-weight affinity eluent for the separation of phospho compounds [1,2].

The elution of the phospho compounds under various conditions is only a part of the total problem. Further studies of the mechanisms of the different binding interactions of iron(III) to proteins are essential to achieve a practical technique for the separation of proteins with respect to their different types of interactions. For affinity eluents directed to other than the iron(III)–phosphate interactions, the approaches based on different types of affinity and/or various chromatographic conditions must be investigated. The interaction between carboxyl groups and chelated iron(III) ions is very weak compared with the phosphate–iron(III) interactions. However, when there are clusters of both carboxylic and phenolic groups, the chelating effect is increased, becoming comparable to that for phosphate groups. It is possible that by varying the type of salt and its concentration one could enhance the difference in the adsorption strength between phosphoproteins and other proteins. Another approach may involve the inclusion of specific modifiers directed to either phosphate or carboxyl and phenolic groups in order to use them for affinity elution of compounds carrying different numbers of phosphate, carboxyl and phenolic groups. To develop such procedures more information about the way in which specific eluents affect the elution profiles of different compounds is desirable. The possibility of directing the interaction toward a single kind of adsorption on metal-chelated gels provides excellent opportunities for FPLC.

Iron(III)–chelated Superose used together in the FPLC system enables analytical studies on the chromatographic behaviour of different molecules. Moreover, the chromatography on iron(III)-chelated Sepharose, used in regular IMAC, and on iron (III)-chelated Superose was nearly identical, indicating that both systems can be readily compared.

ACKNOWLEDGEMENTS

We are grateful to Dr. Eugene Sulkowski for his critical comments. We are also indebted to Miss Ju-

dith Scoble for linguistic revision and to Mrs. Inga Johansson for help in preparing the manuscript. This work was financially supported by the Swedish Board for Technical Development, Nordic Industrial Fund and Erna and Victor Hasselblad's Foundation.

REFERENCES

1 L. Andersson and J. Porath, *Anal.Biochem.*, 154 (1986) 250.
2 G. Muszyńska, L. Andersson and J. Porath, *Biochemistry*, 25 (1986) 6850.
3 H. P. Michel and J. Bennett, *FEBS Lett.*, 212 (1987) 103.
4 G. Muszyńska, in T. W. Hutchens (Editor), *Protein Recognition of Immobilized Ligands*, Alan R. Liss, New York, 1989, p. 279.
5 H. Flotow, P. R. Graves, A. Wang, C. J. Fiol, R. W. Roeske and P. J. Roach, *J. Biol. Chem.*, 265 (1990) 14264.
6 G. Dobrowolska, G. Muszyńska and J. Porath, *J. Chromatogr.*, 541 (1990) 333.
7 E. Sulkowski, *Makromol. Chem., Macromol. Symp.*, 17 (1988) 335.
8 G. Chaga, L. Andersson, B. Ersson and J. Porath, *Biotechnol. Appl. Biochem.*, 11 (1989) 424.
9 N. Ramadan and J. Porath, *J. Chromatogr.*, 321 (1985) 93.
10 J. Porath, *J. Chromatogr.*, 510 (1990) 47.
11 M. Belew and J. Porath, *J. Chromatogr.*, 516 (1990) 333.
12 F. Marchiori, F. Meggio, O. Marin, G. Borin, A. Calderan, P. Ruzza and L. A. Pinna, *Biochim. Biophys. Acta*, 971 (1988) 332.
13 M. J. Zoller, A. R. Kerlavage and S. S. Taylor, *J. Biol. Chem.*, 254 (1979) 2408.
14 G. Dobrowolska, E. Ber and G. Muszyńska, *J. Chromatogr.*, 376 (1986) 421.
15 G. Dobrowolska, F. Meggio and L. A. Pinna, *Biochim. Biophys. Acta*, 931 (1987) 188.
16 M. M. Bradford, *Anal. Biochem.*, 72 (1976) 248.
17 C. H. W. Hirs, S. Moore and W. H. Stein, *J. Biol. Chem.*, 219 (1956) 623.
18 D. H. Spackman, W. H. Stein and S. Moore, *Anal. Chem.*, 30 (1958) 1190.
19 P.T. Tuazon, E. W. Bingham and J. A. Traugh, *Eur. J. Biochem.*, 94 (1979) 497.
20 G. M. Hathaway and J. A. Traugh, in E. Stadtman and B. Horecker (Editors), *Current Topics in Cellular Regulation*, Academic Press, New York, 1982, p. 101.
21 H. N. Bramson, E. T. Kaiser and A. S. Mildvan, *CRC Crit. Rev. Biochem.*, 15 (2) (1984) 93.
22 J. Porath, *J. Mol. Recogn.*, 3 (1990) 123.
23 F. Hofmeister, *Naunyn-Schmiedebergs Arch. Pathol.*, 24 (1888) 247.