Selective Adsorption of Phosphoproteins on Gel-Immobilized Ferric Chelate†

Grażyna Muszyńska,‡ Lennart Andersson,§ and Jerker Porath*,§

Institute of Biochemistry, University of Uppsala, Biomedical Center, S-751 23 Uppsala, Sweden, and Institute of Biochemistry and Biophysics, Polish Academy of Sciences, 02-532 Warsaw, Poland

Received February 5, 1986; Revised Manuscript Received May 15, 1986

ABSTRACT: Ferric ions are very strongly adsorbed to iminodiacetic acid substituted agarose. This firmly immobilized complex acts as a selective immobilized metal affinity adsorbent for phosphoproteins. Chromatography based on this principle is illustrated by the adsorption–desorption behavior of egg yolk phosvitin before and after dephosphorylation as well as by the change in the chromatographic pattern before and after enzymic phosphorylation of selected histones. The strength of binding is dependent on the phosphate content. The difference in binding before and after phosphorylation of a single amino acid residue is demonstrated. Affinity elution can be accomplished by inclusion in the buffer of (1) phosphoserine or (2) a displacing metal ion such as Mg^{2+}.

Intracellular phosphorylation plays an important role in various cellular processes (Hathaway & Traugh, 1982; Bradbury, 1976; Krebs & Beavo, 1979; Welles, 1979). However, in vitro studies of protein phosphorylation are difficult to control since protein kinases, phosphatases, and endogenous substrates interfere with the determination of the phosphoproteins. Therefore, a method for specific separation of the latter is highly desirable. In a preliminary publication, Andersson and Porath (1986) described the use of ferric ions immobilized on IDA–agarose to fractionate ovalbumin and pepsin into components that differ in their phosphorylation states. We report here our evaluation of both the quantitative and qualitative contributions of phosphoserine groups toward the differential adsorption and desorption of these proteins on immobilized ferric ions. Two model proteins with widely different degrees of phosphorylation have been used in our study.

Experimental Procedures

Materials. Phosvitin (egg yolk), histone type II-AS (calf thymus), protein kinase catalytic subunit (bovine heart), alkaline phosphatase (bovine intestine), ATP, DTE,[1] BSA, *O*-phospho-DL-serine, MES, PIPES, Tris, and pNPP (Sigma 104 phosphatase substrate) were from Sigma (St. Louis, MO). 1-Amino-2-naphthol-4-sulfonic acid was from Hopkin and Williams Ltd. (London, England). Chelating Sepharose Fast Flow was a gift from Pharmacia Fine Chemicals (Uppsala, Sweden). [γ-^{32}P]ATP was from Amersham International (Amersham, Bucks, England). The salts (pro analyis grade) were obtained from the following sources: magnesium and sodium chlorides, Kebo Laboratories (Stockholm, Sweden); ferric chloride, Riedel de Haen AG (Seetze bei Hannover, GFR); sodium bisulfite, Fisher Scientific Co. (New York); sodium sulfite, Hopkin and Williams Ltd. (Essex, England); potassium phosphate, J. T. Baker Chemical Co. (Phillipsburg, NJ); and ammonium molybdate, Mallinckrodt Chemical Works (St. Louis, MO).

Methods. Partial dephosphorylation of phosvitin was accomplished by incubation of 10 mg of phosvitin with 3.5 units of intestinal alkaline phosphatase in 10 mM Tris-HCl, pH 8.1, at 30 °C in a final volume of 2 mL. After 19 h of incubation, the sample was adjusted to pH 6.0 and applied to the Fe^{3+}-IDA column.

Determination of Phosphatase Activity. The phosphatase activity of various fractions was determined with 100-μL aliquots in 1 mL of 1.5 M Tris-HCl, pH 8.0, and 0.5 mL of 0.003 M pNPP. After reaction at room temperature for 10 min, the activity was determined from absorption measurements at 410 nm.

Phosphorylation of the Major Histone Fraction Eluted from the Fe^{3+}-IDA Gel at pH 7.2. The histone fraction (6 optical units at 280 nm) was incubated with 2000 nmol of ATP (containing 2.5 μCi of [γ-^{32}P]ATP) and the catalytic subunit of protein kinase (1 unit/nmol of ATP) previously activated by DTE according to the manufacturer's suggestions. The incubation (4-mL sample) was performed in the presence of 50 mM Tris-HCl (pH 7.4) and 10 mM $MgCl_2$ at 30 °C for 18 h. The reaction was stopped by removal of ATP by using small columns (PD-10) of Sephadex G-25-M (Pharmacia, Uppsala, Sweden). The counted radioactivity indicated that 36% of the total radioactivity was incorporated into the protein. After concentration, 2 mL of the phosphorylated histone (about 2.7 optical units) was loaded onto a Fe^{3+}-IDA column in 50 mM MES, pH 6.0, containing 1 M NaCl. Total amino acid analysis was carried out as described previously (Hirs et al., 1956; Spackman et al., 1958).

Protein concentrations were monitored by absorbance measurements at 280 nm and/or according to the colorimetric procedure of Bradford (1976) using BSA as standard. The content of protein-bound phosphate was determined according to Bartlett (1959) using potassium phosphate as standard. Concentration of protein solutions was performed by using a Centricon disposable microconcentrator equipped with a M_r 10 000 cutoff membrane (Amicon Corp., Danvers, MA).

† Financial support was obtained from The Swedish Natural Science Research Council, The Swedish Board for Technical Development, the Erna and Victor Hasselblad Foundation, and The Polish Academy of Sciences.

‡ Polish Academy of Sciences.
§ University of Uppsala.

[1] Abbreviations: IMA, immobilized metal affinity; IDA, iminodiacetic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid; TCA, trichloroacetic acid; BSA, bovine serum albumin; pNPP, *p*-nitrophenyl phosphate; DTE, dithioerythritol; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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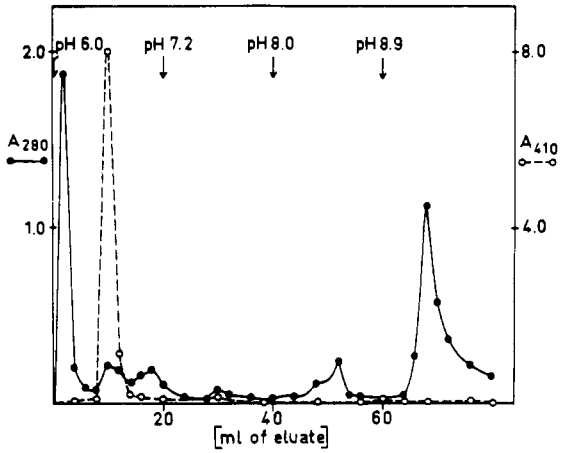

FIGURE 1: Elution of dephosphorylated phosvitin on Fe^{3+}-IDA gel using a stepwise pH gradient: (●) absorbance at 280 nm; (○) alkaline phosphatase activity (ΔA_{410}/10 min).

Table I: Phosphate Determination of Dephosphorylated Phosvitin Eluted from Fe^{3+}-IDA Gel

fractions eluted at pH	nmol of protein for phosphate determination	nmol of phosphate determined	mol of phosphate/mol of protein
6.0[a]	6.75	33.0	4.89
8.0	10.99	93.0	8.46
8.9	8.76	290.0	33.10

[a] The fractions containing the alkaline phosphatase activity (see Figure 1) were excluded from the phosphate determination. Results are expressed as the averages from three independent experiments.

Chromatography. Chelating Sepharose 6B (IDA–agarose) was packed in columns (1 × 6 cm, V_t about 5 mL) in distilled water and charged with 4 bed volumes of 50 mM ferric chloride. For removal of the excess or unbound and loosely bound metal, the column was thoroughly washed with 5 volumes of the following solutions: water, 50 mM Tris-HCl, pH 8.9, and equilibration buffer of 50 mM MES, pH 6.0. Two-milliliter aliquots of phosvitin (10 mg) in 50 mM MES, pH 6.0, or histone (25 mg, 9.0 optical units) in 50 mM MES, pH 6.0, containing 1 M NaCl were applied to each column. Two-milliliter fractions were collected (room temperature) at 12 mL/h by using the following buffers (20 mL) for phosvitin: (1) 50 mM MES/NaOH, pH 6.0; (2) 50 mM PIPES/HCl, pH 7.2; (3) 50 mM Tris/HCl, pH 8.0; (4) 50 mM Tris/HCl, pH 8.9. For elution of the histones the same buffers (20 mL each) containing 1 M sodium chloride were used. Soon after each experiment the columns were regenerated with 0.2 M EDTA and washed with redistilled water. The metal-free columns were stored at room temperature and charged with ferric ions directly before use. Each column was reused several times.

RESULTS

Chromatographic Behavior of Phosvitin in the Native State and after Dephosphorylation. Phosphoserine residues account for more than half of the amino acid composition of egg yolk phosvitin. This protein therefore represents one of the most highly phosphorylated proteins known (Taborsky, 1974). We found this model protein to interact strongly with the Fe^{3+}-IDA gel. After treatment of phosvitin with alkaline phosphatase, the elution pattern changed considerably. Fractions eluting at pH 6, 8, and 8.9 were pooled, concentrated, desalted, and analyzed for phosphate. The protein that eluted at pH 8.9 (Figure 1) contained 33 phosphorylated amino acid residues (Table I). The proteins eluting at pH 8 and 6 contained only about 8 and 5 phosphorylated amino acid residues, respectively.

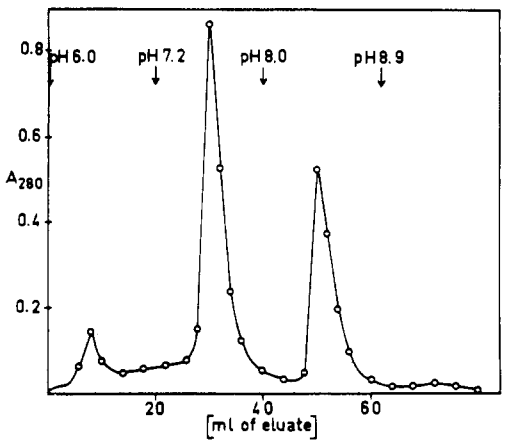

FIGURE 2: Chromatography on Fe^{3+}-IDA gel of histone mixture using stepwise pH changes.

Table II: Amino Acid Composition of Histone Fractions Separated on Fe^{3+}-IDA Gel[a]

amino acid	separated fraction mixture A	B	C	phosphorylated fraction B separated by gradient $MgCl_2$ peak I	peak II
Asp	2.81	5.66	4.77	4.96	5.76
Thr	5.89	5.00	6.25	5.22	4.91
Ser	6.28	7.22	7.25	8.27	7.30
Glu	4.90	8.53	8.58	6.52	8.65
Pro	7.87	4.66	4.58	7.52	4.11
Gly	7.76	8.22	8.16	9.86	8.24
Ala	21.76	12.05	11.75	14.55	11.97
Val	5.12	6.63	6.80	6.14	6.66
Met	0.28	0.65	0.54	0.00	0.74
Ile	1.92	4.33	4.48	2.14	4.28
Leu	5.73	8.45	8.56	4.90	3.32
Tyr	1.12	3.19	3.29	1.21	3.32
Phe	1.04	1.27	1.28	1.12	1.20
His	0.45	2.57	2.58	1.55	2.36
Lys	22.67	13.39	12.99	20.08	13.16
Arg	4.40	8.15	8.38	6.00	8.75

[a] Tryptophan and cystine were not determined. Norleucine (400 nmol) was added as internal standard for amino acid analysis.

Chromatographic Behavior of Histones. (*A*) *Before Phosphorylation.* Chromatography of the histone mixture on the Fe^{3+}-IDA gel resolves three peaks of protein (Figure 2). Proteins eluting at pH 6.0, 7.2, and 8.0 will be referred to as fractions A, B, and C, respectively. Amino acid compositions (Table II) suggest that, according to the nomenclature of Bradbury (1976), fraction A mostly contains the lysine-rich histone H1, fraction B predominantly contains the slightly lysine-rich histone H2a, and fraction C consists of a mixture of histones including H3 and H2a (Hnilica, 1975; Elgin & Weintraub, 1975).

(*B*) *After Phosphorylation.* To determine the effects of specific phosphorylation on the relative affinity of proteins for the Fe^{3+}-IDA gel, the catalytic subunit of cAMP-dependent protein kinase was used to selectively phosphorylate a subfraction of the histone proteins. Fraction B (eluting at pH 7.2), which represents approximately 60% of the total recovered protein, was phosphorylated by using [γ-^{32}P]ATP and the catalytic subunit of protein kinase. Under the experimental conditions outlined, approximately 1.4 amino acid residues were phosphorylated per mole of protein, assuming a molecular weight of 14 000. Phosphorylation of fraction B led to an increase in the pH required for elution from pH 7.2 to pH 8, as shown in Figure 3. Since all of the histone fractions were

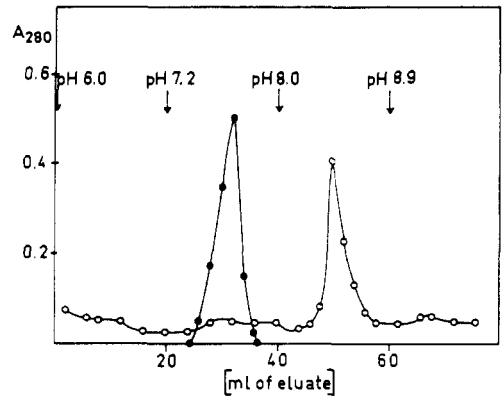

FIGURE 3: Difference in the pH-dependent desorption between phosphorylated (O) and nonphosphorylated histone fraction B (●) from Fe^{3+}-IDA gel.

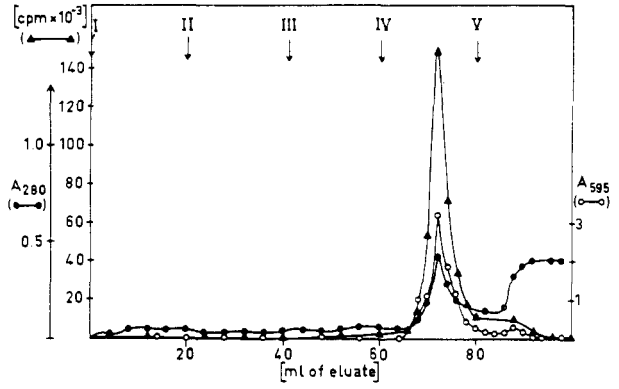

FIGURE 4: Elution of phosphorylated histone fraction B from Fe^{3+}-IDA gel by phosphoserine at (I) 0, (II) 5, (III) 10, (IV) 25, and (V) 50 mM: (●) ΔA_{280}; (O) ΔA_{595}; (▲) radioactivity.

chromatographed on the Fe^{3+}-IDA gels in the presence of 1 M NaCl, simple electrostatic interactions cannot account for the differential elution of fraction B before (pH 7.2) and after (pH 8) phosphorylation. Further increases in the sodium chloride concentration up to 4 M had no influence on the desorption of the phosphorylated fraction B at pH 7.2 (data not shown).

Affinity Elution. After phosphorylation fraction B is tightly bound to the adsorbent at pH 7.2 (Figure 3) but can be effectively desorbed by including phosphoserine in the buffer (Figure 4). The protein is eluted readily by 25 mM phosphoserine, a fact that indeed indicates the specificity of the displacement. Some leakage of Fe^{3+} ions, approximately 0.1 mM, was observed to interfere with measurement of UV absorption at 280 nm. Therefore, protein was also determined according to Bradford (1976).

Various metal ions presumed to have affinities for phosphate groups were attempted as specific eluting agents. $LaCl_3$ and $MgCl_2$ can be dissolved to at least 1 M concentration and brought to pH 7.2 without the formation of insoluble hydroxides. While Mg^{2+} was effective as an eluant, La^{3+} was not. Mn^{2+} and Fe^{3+} salts could not be studied due to low solubility of the hydroxides. Two main peaks were obtained with a continuous concentration gradient of $MgCl_2$ (Figure 5). The proteins in peaks I and II were found to differ in amino acid composition (Table II). Radioactivity measurements suggest that the peak II material is more extensively phosphorylated than that of peak I. It was estimated that the degree of phosphorylation was 0.9 and 1.9 mol of phosphate/mol of protein, respectively. Those figures are relative and approximate, but the ratio of phosphate contents in the components is reliable.

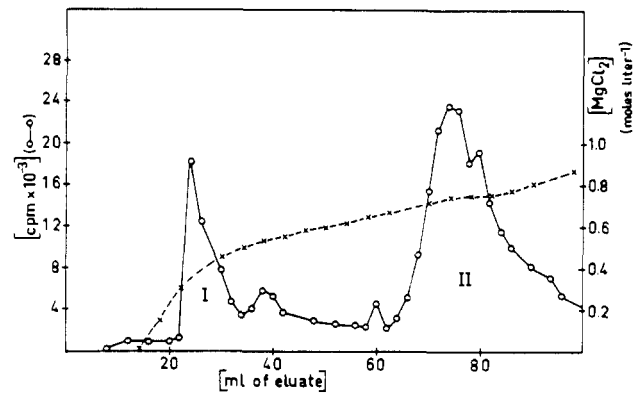

FIGURE 5: Gradient elution of phosphorylated histone fraction B (O) from Fe^{3+}-IDA gel using a continuous gradient of magnesium chloride (×).

DISCUSSION

Ferric ions immobilized on chelating gels display considerable specificity as demonstrated by the retention of certain serum proteins [Porath and Olin (1983) and Ramadan and Porath (1985); see also Andersson and Porath (1986)]. Since immobilized metal ions may also act as ion exchangers, the present investigation was designed to further study the nature of the protein phosphate–immobilized Fe^{3+} interaction by appropriately designed chromatography. Furthermore, an additional goal of this study was to develop procedures for the affinity purification of phosphoproteins, including their selective desorption using affinity eluants. We have therefore examined the overall contribution of multiple phosphate groups as well as the contribution of one or two phosphate groups to the retention of different proteins on an immobilized ferric chelate.

Phosvitin, the most highly phosphorylated protein known (Taborsky, 1974), is composed of about 220 amino acid residues (Byrne et al., 1984), 56% of which are phosphorylated (Allerton & Perlmann, 1965). This protein is strongly adsorbed by the ferric IDA gel. Dephosphorylation of phosvitin by alkaline phosphatase led to considerable changes in its chromatographic behavior. Extensively dephosphorylated phosvitin was desorbed at considerably lower pH (6.0 and 8.0). These results confirm our prediction that the binding of a phosphorylated protein to the Fe^{3+}-IDA gel is directly correlated with its phosphate content, in the range of 5–33 mol of P/mol of protein.

To evaluate in more detail the contribution of phosphate groups to the protein affinity for the Fe^{3+}-IDA gel, a model protein was chosen in which phosphorylation could be limited to a single residue. Histones belong to the groups of nuclear proteins that are known to undergo specific phosphorylation by cAMP-dependent protein kinase (Langan, 1968). Localization of the phosphorylated sites led to the conclusion that Ser^{38} is phosphorylated in the H1 histone, Ser^{19} and/or Ser^{18} in H2a histone, and Ser^{14} and Ser^{36} in H2b histone (Shlyapnikov et al., 1975). Amino acid analysis data have shown that histone H2a is the main fraction of total histones isolated initially by Fe^{3+}-IDA chromatography. When it is specifically phosphorylated by the catalytic subunit of cAMP-dependent protein kinase, it has a higher affinity for the Fe^{3+}-IDA gel. Since these analyses performed in the presence of 1–4 M NaCl, nonspecific electrostatic interactions could not be the major factor responsible for the pH increase required for elution.

Further evidence for the specific nature of the increased affinity of the phosphorylated histone is derived from our discovery of magnesium ions and phosphoserine as affinity

eluants. Quantitative elution of the phosphorylated fraction of the histone from Fe^{3+}-IDA gel by 25 mM phosphoserine demonstrates the specificity in the interaction between phosphoprotein and the affinity adsorbent. As all phosphorylation reactions are Mg^{2+} ion dependent, it is logical to use magnesium-containing eluants for specific elution. As expected, magnesium ion buffer was effective as a specific eluant. Two proteins with different degrees of phosphorylation were separated by stepwise or linear increases of Mg^{2+} ion. These data suggest that the affinity elution by Mg^{2+} might be used routinely for separation of component proteins carrying different numbers of phosphorylated residues.

It must be pointed out that for any particular system the appropriate conditions for selective adsorption and elution on Fe^{3+}-IDA gel have to be ascertained.

The results described here may be very useful for identification and characterization of phosphoproteins and phosphopeptides derived in vivo and in vitro phosphorylation and dephosphorylation reactions, including oncogen products.

ACKNOWLEDGMENTS

We are indebted to Drs. T. William Hutchens and David Eaker for discussions and linguistic suggestions.

Registry No. $MgCl_2$, 7786-30-3; L-phosphoserine, 407-41-0.

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