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Recognition of phosphate groups by immobilized aluminium(III) ions

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ABSTRACT

Aluminium ions, immobilized on iminodiacetate-agarose, were used as a chromatographic adsorbent. At pH 6.0, in strong salt solution, phosphoamino acids, phosphopeptides, phosphoproteins and nucleotides that contained terminally bound phosphate were retained. At pH 7.5 most of the phosphorus compounds were desorbed. Conversely, phosphate-free compounds were unretained at both pHs and the same behaviour was displayed by nucleotides which contained only internally bound phosphate. These findings might be useful as a basis for the development of a procedure for isolating phosphoproteins.

INTRODUCTION

Studies of the chromatographic behaviour on immobilized Cu(II), Ni(II), Zn(II) and Co(II) ions of selected thiol-free model proteins have demonstrated that surface histidyl groups are the primary electron donors in the interaction between metal and protein [1]. Other metals, such as Fe(III) and especially Al(III), can be assumed to have a higher affinity for oxygen as the electron donor [2]. Chromatography of model compounds on immobilized Fe(III) seems to support this hypothesis. For instance, phosphate compounds are strongly bound, presumably via oxygen [3,4]. Immobilized Al(III) has limited use for the separation of biological molecules. The free metal ion is known to form strong complexes with phosphate, citrate and other oxygen-rich compounds [5]. This property is the basis upon which a new separation method has been attempted. This paper describes the properties of Al(III) immobilized on agarose gel and its possible utility as an adsorbent for phosphorus-containing compounds.

EXPERIMENTAL

Iminodiacetic acid(IDA)-agarose (Chelating Sepharose Fast Flow) was obtained from Pharmacia–LKB (Uppsala, Sweden), or was prepared in this laboratory [6]. The Cu(II)-binding capacity was 30 and 70 μmol/ml wet gel, respectively. A glass column (20 × 10 mm I.D.) containing Chelating Superose was purchased from Pharmacia. The gel consists of the high-performance matrix Superose 12 onto which iminodiaetic acid is coupled. Carboxymethylaspartyl-agarose (CM-asp-agarose) was

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described previously [7] and tris(carboxymethyl)ethylenediamine (TED)-agarose were synthesized according to a previous report [8]. Binding capacities were 42 and 90 μmol of Cu ions per millilitre, respectively. However, CM-asp-agarose displayed poor aluminium binding and was not studied further. Aluminium binding to TED-agarose was strong but neither the model compounds, *i.e.*, phosphoprotein and phosphoamino acids, nor the other proteins used in this work were bound to TED–Al(III)–agarose. Those results confirm some observations made earlier [9].

Ovalbumin, pepsin (4300 U/mg protein), pepsinogen (porcine stomach, grade 1-S, 3900 U/mg protein), rabbit muscle phosphorylase *a* and phosphorylase *b* were purchased from Sigma (St. Louis, MO, U.S.A.). Chicken muscle glycogen phosphorylase was prepared following the procedure of Petell *et al.* [10]. Assays for phosphorylase *a* and phosphorylase *b* activity were carried out according to the procedure of Hedrick and Fischer [11], slightly modified. It was found that the chicken muscle extract contained high phosphorylase *b* activity. Alkaline phosphatase (calf intestine, grade I) was obtained from Boehringer (Mannheim, Germany). Ovalbumin was fractionated on immobilized IDA–Fe(III) into components containing no, one and two phosphate groups, according to an earlier report [3]. L-Amino acids, phospho-L-serine, phospho-L-threonine, phospho-L-tyrosine, nucleotides and nucleotide derivatives were of analytical-reagent grade. The synthetic peptides Arg–Arg–Ala–Ser–Val–Ala and its phosphorylated analogue Arg–Arg–Ala–P–Ser–Val–Ala were a gift from Dr. B. Fransson of this institute.

The aluminium concentration in the column eluates was determined according to a procedure in which the metal is complexed with Alizarin S [12]. Dephosphorylation of ovalbumin and pepsinogen was done by incubation with alkaline phosphatase (1–2 units/mg protein). Protein samples were analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) on microplates (Phast System, Pharmacia), or by electrophoresis in 1% agarose gel slabs. Staining was done in solutions of Coomassie Brilliant Blue.

Preparation of columns and chromatography

Metal-free gel was suspended in water, degassed and poured into a chromatographic column (60 × 10 mm I.D., bed volume 5 ml). Columns were charged with a solution of aluminium chloride (50 m*M*) in distilled water. The charging was considered to be complete when a precipitate of aluminium hydroxide was formed from a small aliquot of the eluate after addition of one drop of 5% sodium carbonate solution. After washing out unbound metal with three column volumes of water, the column was equilibrated with five column volumes of starting buffer. Freeze-dried and salt-free samples were dissolved in starting buffer; in some instances they were desalted in this buffer on a small Sephadex G-25 column prior to chromatography. Runs were made at room temperature with a flow-rate of 15–20 ml/h. Fractions of 1.0 or 1.5 ml were analysed for protein and enzymic activity. The metal was removed after each run with 0.1 *M* EDTA (0.5 *M* NaCl; pH 7) and occasionally with a portion of 0.1 *M* sodium hydroxide. Chromatographic runs on Chelating Superose were accomplished using an LKB high-performance liquid chromatographic system consisting of pump (Model 2150), a controller (Model 2152) and a two-channel recorder (Model 2210).

The following buffer systems were used, unless indicated otherwise: pH 5.0, 0.05

M acetic acid–NaOH (1 *M* NaCl); pH 6.0, 0.05 *M* MES–NaOH (1 *M* NaCl); pH 7.0, 0.05 *M* PIPES–HCl (1 *M* NaCl); and pH 8.0, 0.05 *M* Tris–HCl (1 *M* NaCl), where MES = 4-morpholinoethanesulphonic acid and PIPES = 1,4-piperazinodiethanesulphonic acid.

RESULTS AND DISCUSSION

Aluminium binding and stability of the chelating gel

The binding capacity was determined by frontal analysis. The copper- and aluminium-binding capacities were 30 and 14 μmol per millilitre wet gel, respectively, for Chelating Sepharose or Superose and 70 and 32 μmol/ml, respectively, for the "home-made" IDA-agarose. The stability of the metal chelates was checked by measuring the aluminium ion concentration at pH 5.0, 6.0, 7.0 and 8.0 in eluates from a column that was completely loaded with metal. The leakage from Chelating Sepharose was found to be 0.015, <0.004, <0.004 and 0.020 μmol/ml, respectively, which indicates good stability in this pH range. From TED-agarose it was less than 0.004 μmol/ml eluate at these pH values.

Chromatography of low-molecular-mass compounds

Amounts of 0.5–1 mg of compounds containing bound phosphate and phosphate-free analogues, amino acids, nucleosides, nucleotides and peptides were dissolved in starting buffer and chromatographed, one by one, on Al(III)–IDA-agarose at different pH values. The results are given in Table I. Evidently none of the phosphate-free substances was retained. All the substances that were retained at pH 6.0 (1 *M* NaCl) contain an external (terminal) phosphate group. The absence of retention of GpU, ApG, NAD and UDPG indicates that internal phosphate groups do not contribute significantly to adsorption. The increased affinity of ADP, GTP and ATP might be due to metal chelation. Strong chelating of Al(III) ions to ADP has been reported [2]. Compounds adsorbed on Al(III)–IDA–agarose and containing mono-esterified phosphate group(s) could be desorbed either by increasing the pH to 8 or by adding 20 m*M* phosphate. ADP, ATP and GDP, once adsorbed, resisted desorption at pH 8 or 9, but their elution proceeded reasonably well at pH 11.

Chromatography of model proteins

Porcine pepsinogen and pepsin are single-chain polypeptides composed of 370 and 329 amino acid residues, respectively. In each protein there is a single phosphorylated serine that is positioned fairly near the amino-terminus. On activation, pepsinogen loses its 41-residue N-terminal peptide. Both pepsinogen and pepsin were weakly retained in MES buffer (pH 6.0) (1 *M* NaCl). The retention became strong if 0.5 *M* potassium phosphate was substituted for 1 *M* sodium chloride. Both enzymes were desorbed with good recoveries ($<90\%$ for pepsinogen, 70% for pepsin) by 20 m*M* phosphate buffer (0.5 *M* K_2SO_4). In a control experiment, dephosphorylated pepsinogen was run under similar conditions. Fig. 1 shows that pepsinogen was strongly retained but the dephosphorylated enzyme unretained at pH 6. Presumably, little or no conformational change takes place after the dephosporylation because there is no significant loss in activity. These results suggest that immobilized Al(III) can recognize a seryl-phosphate group among more than 300 residues.

TABLE I

RETENTION OF LOW-MOLECULAR-MASS SUBSTANCES ON Al(III) IONS IMMOBILIZED ON CHELATING SEPHAROSE FAST FLOW

Substance[a]	pH of buffers in 1 *M* NaCl			
	5.0	6.0	7.0	8.0
L-Amino acids	–	–	–	–
Phosphoserine	+	+	–	–
Phosphothreonine	+	+	–	–
Phosphotyrosine	+	+	–	–
Arg–Arg–Ala–Ser–Val–Ala	–	–	–	–
Arg–Arg–Ala–P–Ser–Val–Ala	+	+	–	–
Nucleosides	–	–	–	–
AMP	+	+	+	–
ADP	+	+	+	+ –
ATP	+	+	+	+ –
GMP	+	+	+	–
GDP	+	+	+	+ –
CMP	+	+	+	–
NAD^+	n.d.	–	–	–
NADP	n.d.	+	–	–
FAD	–	–	–	–
GpU	–	–	–	–
ApG	–	–	–	–
UDPG	–	–	–	–
cAMP	–	–	–	–

[a] Standard abbreviations; also GMP = guanosine 5′-phosphate; CMP = cytidine 5′-monophosphate; NADP = nicotinamide adenine dinucleotide phosphate; FAD = flavin adenine dinucleotide; GpU = guanosine phosphate uridine; ApG = adenosine phosphate guanosine; UDPG = uridine diphosphate glucose; cAMP = cyclic adenosine 3′,5′-monophosphate.

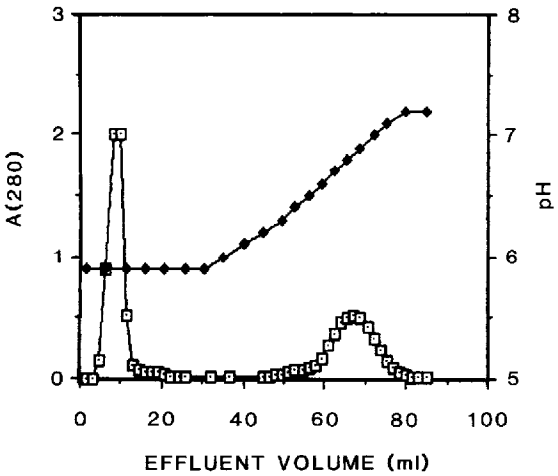

Fig. 1. Chromatography of pepsinogens on Al(III)–IDA γ-agarose. A sample of 4 mg of pepsinogen in 5 ml was applied and the column was washed with 45 ml of 0.05 *M* MES–NaOH (0.5 *M* K_2SO_4) (pH 6.0). The column was then rinsed with 20 m*M* phosphate buffer (0.5 *M* K_2SO_4) (pH 7); the buffer change is indicated by an arrow. □ = Porcine pepsinogen; ◆ = dephosphorylated porcine pepsinogen.

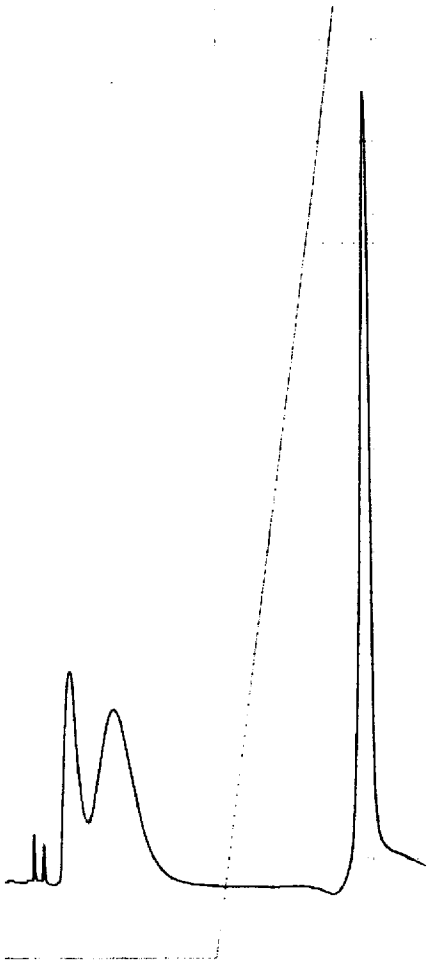

Fig. 2. Recorder trace from chromatography of ovalbumin on Al(III)–IDA-Superose column. 2 mg of ovalbumin were dissolved in 0.05 *M* MES–NaOH (1 *M* NaCl) buffer (pH 6.0) and injected into the column. The column was then rinsed with starting buffer and developed with a pH gradient formed by mixing starting buffer with 0.05 *M* PIPES–HCl (1 *M* NaCl) buffer (pH 7.2). The flow-rate was 0.25 ml/min. Time scale: 1 cm = 10 min.

Native ovalbumin was chromatographed on Al(III)–Chelating Superose. A 2-mg amount was dissolved in MES buffer (pH 6.0) (1 *M* NaCl) and applied. After washing out weakly bound protein, the column was developed with a pH gradient from 6.0 to 7.3. The results are illustrated in Fig. 2. Purified ovalbumins with no, one and two phosphate groups were chromatographed, one by one, in the same way. By comparison, the first peak in Fig. 2 was identified as ovalbumin with no phosphate, the second peak as the monophosphate ovalbumin and the third peak as the diphosphate ovalbumin. The poor retention of the monophosphate ovalbumin might be due to partial masking of the phosphate group. The resolution was greatly dependent on the

flow-rate, which suggests slow desorption kinetics. The overall protein recovery was always near 100%. It was found, by analysis, that diphosphate ovalbumin scavenged some aluminium from the adsorbent.

Muscle glycogen phosphorylase may exist in the form of phosphorylase *a*, which is produced from phosphorylase *b* phosphorylation of one seryl residue per subunit, *A priori*, this enzyme seems rather complicated to choose as a model protein. Both the *a* and *b* forms contain one bound pyridoxal phosphate; however, this group is contained deep within the interior of the protein. Depending on the conditions, the *a* and *b* enzyme may be built up of two four subunits. Phosphorylase *b* was unretained when chromatographed on Al(III)–IDA-agarose under the usual conditions. Phosphorylase *a*, on the other hand, was always adsorbed. However, depending on the enzyme source, the adsorption was sometimes strong and sometimes weak. This variation might be due to the different aggregation and/or conformational states that have been reported [13]. These states are assayed only with difficulty and therefore any further interpretation seems impossible. However, the outcome of these experiments does not contradict the hypothesis that binding occurs via phosphate groups.

Some other model proteins that are known to interact through histidyl groups with Cu(II)– and Ni(II)–IDA-agarose gel were chromatographed using the same procedure. None of them, *i.e.*, horse cytochrome *c*, human serum albumin, whale myoglobin and hen's egg lysozyme was, however, adsorbed at pH 6.0, 7.0 or 8.0. This means that immobilized Al(III) does not recognize one or several surface histidyl side-chains.

Adsorption of proteins in crude extracts

A 3-ml volume of dialysed human serum were chromatographed on Al(III)–Chelating Sepharose at pH 6.0 in the presence of 1 or 2 *M* sodium chloride or 0.5 *M* potassium sulphate. After washing with 25 ml of starting buffer desorption was effected with 20 m*M* phosphate (1 *M* NaCl) (pH 7) and subsequently with 0.1 *M* EDTA (0.5 *M* NaCl). However, only small amounts of protein (0.1–0.5%) were desorbed and therefore no attempt was made to analyse this material. Hen's egg white was filtered and diluted 10-fold with 0.05 *M* MES (1 *M* NaCl) (pH 6.0). A 1-ml volume was applied, the column was washed with buffer and proteins were desorbed with 0.05 *M* Pipes (1 *M* NaCl) (pH 7.2) (Fig. 3a). The desorbed material was analysed by SDS-PAGE and was found to be homogeneous with the same molecular mass as standard ovalbumin (not shown). Agarose gel electrophoresis revealed that the material behaved as diphosphate ovalbumin (Fig. 3b). The experiments on extracts suggest that Al(III)–Chelating Sepharose has a high selectivity for the phosphoprotein.

Rat liver acetone powder (Sigma L 1380) was extracted with MES buffer (pH 6.0) (1 *M* NaCl) at room temperature for 20 min. After centrifugation the extract was applied to the Al(III) column and chromatographed. A large amount of UV-absorbing material was desorbed on increasing the pH. The UV spectrum and protein analysis showed that this material contained a mixture of nucleotides but no protein.

Extracts of chicken muscle were transferred to MES buffer (pH 6.0) (1 *M* NaCl) to remove EDTA and phosphate ions. Ten millilitres were chromatographed on Al(III)–IDA-agarose gel in the same buffer. There was virtually no adsorption of protein and all of the applied phosphorylase b activity appeared in the breakthrough

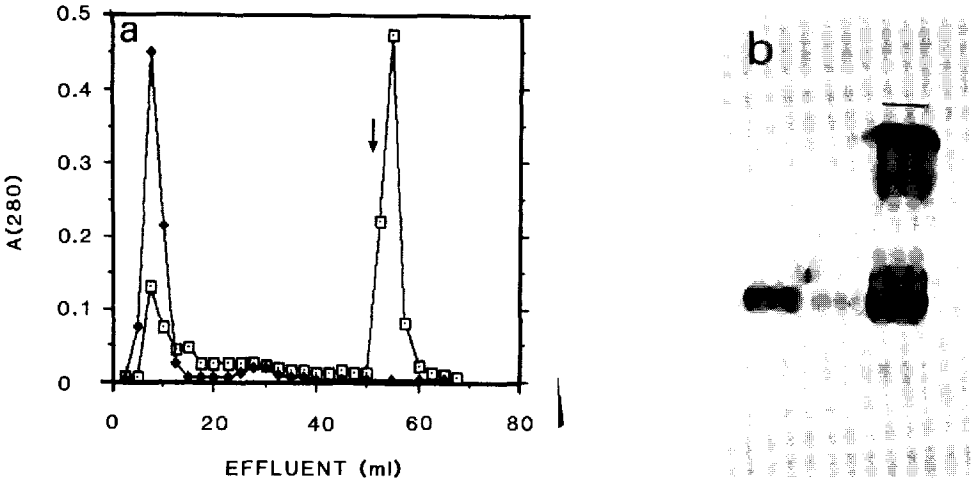

Fig. 3. (a) Chromatography of crude egg white on Al(III)–IDA γ-agarose. A 1-ml sample of diluted egg-white extract was applied and the column was rinsed with 25 ml of starting buffer. The column was then developed with a pH gradient formed by mixing 25 ml of starting buffer with 25 ml of 0.05 *M* PIPES–HCl (1 *M* NaCl) buffer (pH 7.2). Finally, the column was rinsed with the second buffer. (b) Agarose electrophoresis of hen's egg-white proteins. Lanes: 1 = crude extract of egg white; 2 = pooled fractions from peak 2 (at 55 ml) in (a).

peak. In another experiment the extraction procedure was done in the absence of EDTA but in the presence Mn(II) ions. This extract displayed phosphorylase a activity. After chromatography on Al(III)–IDA γ-agarose gel in the same buffer as before, all of the phosphorylase a was found to be adsorbed. It was, however, difficult to recover the enzyme in the usual manner. However, even in this complicated case, immobilized Al(III) appears to recognize bound phosphate groups.

In summary, it seems that aluminium ions can recognize a phosphate group having an external position within a molecule. With proper handling, the adsorbent might be useful for the group isolation of certain compounds, including proteins, containing a small number of externally bound phosphate groups. All evidence in this work suggests that the specificity for phosphorylated protein among other proteins is remarkably high, let alone the possible interference of nucleotides containing an external phosphate group that may be present in biological extracts.

The data from this and from previous studies [3,8,14,15] allow some comparison between the properties of immobilized Fe(III) and immobilized Al(III). Obviously, the latter adsorbent has a superior selectivity for phosphoprotein. On the other hand, binding of phosphorylated proteins to Al(III)–IDA γ-agarose gel seems comparatively weaker and therefore requires the presence of high concentrations of salt. Therefore, it may be advantageous that both adsorbents, combined, be used for the purpose of purifying proteins, especially phosphoproteins.

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