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Model studies on iron(III) ion affinity chromatography

Interaction of immobilized metal ions with nucleotides

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ABSTRACT

Mononucleotides are able to bind with immobilized iron(III) ions at low pH in the presence of 1 *M* sodium chloride and can be desorbed by increasing the pH. All the mononucleotides studied, bound at pH 5.5 to gel-chelated iron(III) ions, were eluted from the adsorbent in the pH range 7.0–7.4. No significant difference was observed in the elution profiles of mono-, di-, tri- and tetraphosphate nucleotides or their deoxy forms. Nucleosides, cyclic mononucleotides and dinucleotides containing all phosphate groups in the internal position do not bind to the immobilized metal ion under these conditions. The results obtained indicate that for interaction of nucleotides with immobilized iron(III) ions, one free terminal phosphate group is responsible.

INTRODUCTION

Immobilized iron(III) ion chromatography has proved to be a promising technique for the selective separation of macromolecules [1–3]. There is evidence that immobilized iron(III) ions interact with phosphate-oxygen and to some extent with other negatively charged groups on peptides and proteins [4]. We have demonstrated that immobilized iron(III) ions can preferentially bind phosphoproteins [1,2] and can be used for the separation of phosphopeptides from a tryptic digest of proteins [5]. Moreover, some data suggest that the strength of binding is dependent on the phosphate content of proteins [2,6].

The aim of this work was to extend the previous studies and to look for a mode of interaction between the immobilized iron(III) and non-protein phosphocompounds. For this purpose nucleotides and their derivatives, differing in the number of phosphate and nucleotide groups, were chosen as models. The results could indicate whether the interaction might be exploited for the rapid and high-yield fractionation of

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nucleotides and nucleic acids by chromatography on iron(III) chelate adsorbent(s) on the basis of phosphate content.

EXPERIMENTAL

Materials

Nucleosides, mono- and dinucleotides, poly C (polycytidylic acid), DNA from calf thymus, morpholinoethanesulphonic acid (MES), Tris and iron(III) chloride were obtained from Sigma. All other chemicals were of analytical-reagent grade.

Buffers

Buffer 1 was 0.05 *M* MES + 1 *M* NaCl (pH 5.5), buffer 2 was 0.02 *M* MES + 1 *M* NaCl (pH 6.5) and buffer 3 was 0.10 *M* Tris + 1 *M* NaCl (pH 7.7).

Chromatography

Degassed chelating Sepharose Fast Flow (Pharmacia–LKB) was packed in columns (1.5 × 1 cm I.D.; $V_t \approx 1$ ml) in distilled water and charged with a few volumes of 20 m*M* iron(III) chloride. For removal of the excess of unbound and loosely bound metal ions the columns were washed with 10–15 volumes of water, buffer 3 and finally equilibration buffer 1. Before chromatography all solutions were degassed. Chromatography was conducted at room temperature with a flow-rate of 15 ml/h and 1.7-ml fractions were collected. In each run 1–2 μmol of an appropriate compound in 0.5 ml of buffer 1 was applied to the column. The column was subsequently washed with 8.5 ml of buffer 1, 8.5 ml of buffer 2 and finally by a continuous pH gradient formed by the gradual mixing of 17 ml of buffer 3 with 17 ml of buffer 2. The eluted compounds were detected by determination of the absorbance at 260 nm, and the pH of the eluate was measured using a pH meter. Other conditions used in some of the separation experiments are indicated in Table I.

Soon after each experiment, the columns were regenerated with 0.1 *M* EDTA–1 *M* sodium chloride and washed with redistilled water. The metal-free columns were stored at room temperature and charged with iron(III) ions directly before use. Each column was reused several times.

RESULTS

As can be seen from Table I, nucleosides (adenosine and guanosine) and cyclic mononucleotides (cAMP and cGMP) are not bound and are eluted from gel-immobilized iron(III) ions by the equilibration buffer at pH 5.5. Dinucleotides bearing one to five internal phosphate groups, polynucleotide (poly C) and DNA are not adsorbed on the metal chelate adsorbent in the presence of 1 *M* sodium chloride. Among the tested constituents of ribonucleic acids, adenine mononucleotides (AMP, ADP and ATP) and guanidine mononucleotides (GMP and Gpppp) bearing one to four phosphate groups are adsorbed on the iron(III)-chelated gel at pH 5.5 and can be eluted by a continuous pH gradient (pH 6.5–7.7) in the presence of 1 *M* sodium chloride (Figs. 1 and 2). A similar elution profile is obtained for deoxymonophosphate nucleotides (dAMP, dGMP, dCMP) (Fig. 3).

The elution patterns of all the mononucleotides studied are similar; purine

TABLE I

CHROMATOGRAPHIC BEHAVIOUR OF NUCLEOTIDES AND RELATED COMPOUNDS ON GEL-IMMOBILIZED IRON(III) CHELATE

Experimental conditions: *ca.* 2–3 optical units at 260 nm of tested compounds in 0.5 ml of buffer 1 were applied to an iron(III)-chelated column ($V_t \approx 0.5$ ml). The column was subsequently washed with 5 ml of buffer 1 and 5 ml of buffer 3 and 1-ml fractions were collected at a flow-rate of 10 ml/h. The compounds detected in the fractions eluted by buffers 1 and 3 were classified as "not bound" and "bound", respectively.

Compound[a]	Chromatographic behaviour	
	Not bound (−)	Bound (+)
Adenosine	−	
cAMP	−	
AMP		+
ADP		+
ATP		+
ApppA	−	
AppppA	−	
cGMP	−	
GMP		+
Gpppp		+
GpG	−	
GpppG	−	
GppppppG	−	
poly C	−	
Deoxy-AMP		+
Deoxy-GMP		+
Deoxy-CMP		+
DNA from calf thymus	−	

[a] Abbreviations: ApppA = p^1,p^3-di(adenosine-5′)triphosphate; AppppA = p^1,p^4 di(adenosine-5′)tetraphosphate; cAMP = adenosine 3′5′-cyclic monophosphate, cGMP = guanosine 3′5′-cyclic monophosphate; Gpppp = guanosine 5′-tetraphosphate; GpG = guanylyl(3′–5′)guanosine; GpppG = p^1,p^3-di(guanosine-5′)triphosphate; GppppppG = p^1,p^5-di(guanosine-5′)pentaphosphate; poly C = polycytidylic acid.

TABLE II

ELUTION OF NUCLEOTIDES FROM GEL-IMMOBILIZED IRON(III) CHELATE

The pH values are taken from Figs. 1–3.

Nucleotide[a]	pH of elution	Nucleotide[a]	pH of elution
AMP	7.30	Gpppp	7.25
ADP	7.40	Deoxy-AMP	7.25
ATP	7.40	Deoxy-GMP	7.25
GMP	7.20	Deoxy-CMP	7.00

[a] For abbreviations, see Table I.

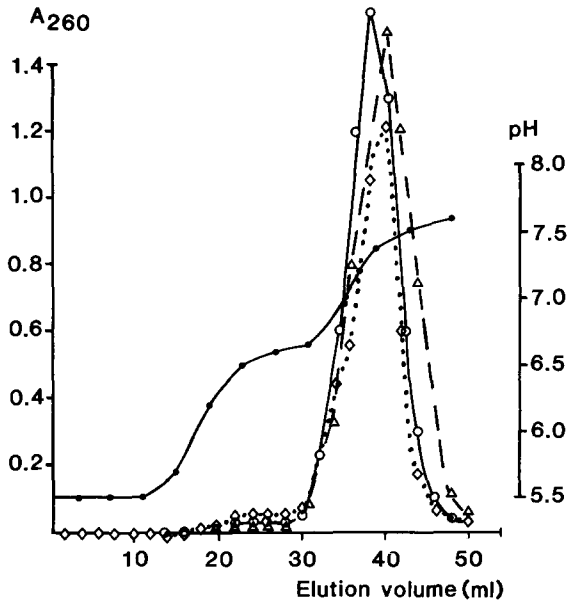

Fig. 1. Elution profiles of adenine nucleotides from Fe^{3+}-chelated gel. ○—○ = AMP; △- -△ = ADP; ◇ · · · ◇ = ATP; ●—● = pH.

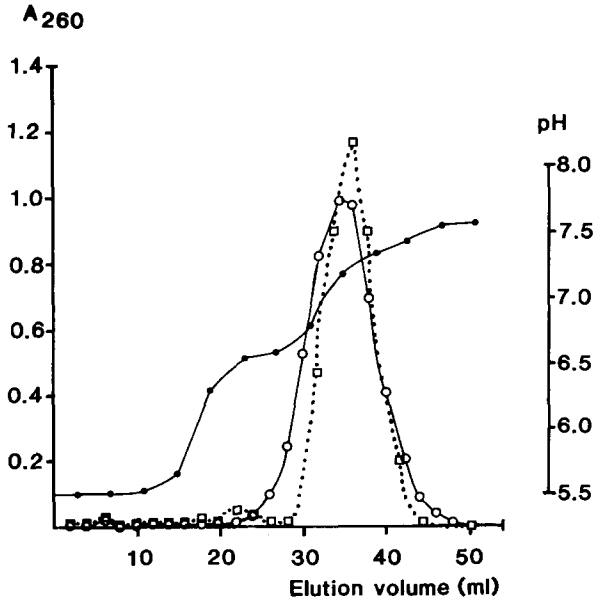

Fig. 2. Elution profiles of guanidine nucleotides from Fe^{3+}-chelated gel. ○—○ = GMP; □ · · · □ = Gpppp; ●—● = pH.

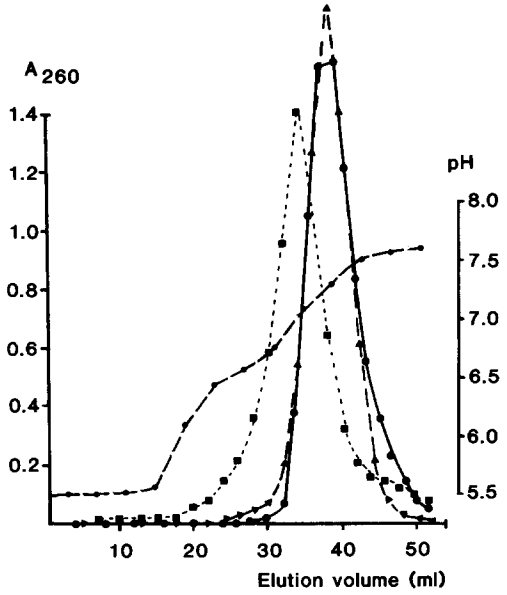

Fig 3. Elution profiles of deoxymonophosphate nucleotides from Fe^{3+}-chelated gel. ●—●, Deoxy-AMP; ▲--▲, deoxy-GMP; ■- -■, deoxy-CMP; ●--● = pH.

mono-, di-, tri- and tetraphosphate derivatives in their ribo and deoxyriboforms are desorbed from gel-immobilized iron(III) ions in the pH range 7.2–7.4, whereas for elution of dCMP a lower pH (7.0) is sufficient (Table II).

DISCUSSION

Immobilized metal affinity chromatography (IMAC) has been extensively applied to the separation and fractionation of proteins. There is evidence that it can also be used for studying the behaviour of nucleic acid constituents. Hubert and Porath [7,8] indicated that pyrimidine nucleotides display weak interaction with immobilized divalent metal ions, such as copper and nickel. The stronger complexation of purine nucleotides on immobilized Cu^{2+} ions allowed their separation from pyrimidine mononucleotides and also provided sufficient selectivity to differentiate AMP from GMP [8]. The number of potential binding sites (particularly nitrogen atoms on the bases, and to a lesser extent the negatively charged oxygen atom in the phosphate residue and possibly the hydroxyl group on the ribose) might account for multiple site attachment with the Cu^{2+} in solution [9,10]. However, under the conditions described by Hubert and Porath [7,8], bases are more strongly adsorbed on immobilized copper ions than the corresponding nucleosides and nucleotides. This seems to exclude any contribution of either the phosphate or the ribose moieties to the binding with the immobilized metal ions.

The present results clearly indicate that for the interaction of nucleotides with gel-immobilized iron(III) ions only free phosphate groups are responsible, because only nucleotides with free terminal phosphate groups are able to bind to such gels. The

strength of binding is not influenced by an increase in the number (one to four) of phosphate groups of nucleotides. The same pH of elution of AMP or GMP and their deoxy homologues indicates the lack of a contribution of the 2′-OH group of ribose to the binding process. However, the slight difference in the chromatographic behaviour of deoxy-CMP compared with deoxypurine analogues suggests that the type of base may play a minor role in the interaction with immobilized trivalent iron.

Cyclic mononucleotides (cAMP and cGMP), where the phosphate group is bound in two positions to ribose (3′ and 5′), do not have any affinity to immobilized iron(III) ions. Also dinucleotides, bearing exclusively one to five internal phosphate groups, are not adsorbed on the column. Biopolymers such as poly C and DNA from calf thymus do not bind to the gel. The most probable explanation is that in large molecules, such as poly C or DNA, one terminal phosphate group is not sufficient for binding to the immobilized metal and/or this group is not very exposed because it is involved in the formation of a higher order structure. In simple molecules, such as cAMP and dinucleotides, even sufficiently exposed phosphate groups, but present as phosphodiesters, are not able to interact with immobilized iron(III) ions. The results suggest that for the formation of a binding site with the immobilized metal, oxygen atoms in the phosphate must be accessible and a phosphomonoester must be present.

The binding of macromolecules to immobilized metal ions involves different types of interactions. Adsorption of proteins to the immobilized metals is typically reinforced at an increased concentration of antichaotropic salts [11], which is probably an indication of metal coordination binding. This work demonstrates that the adsorption of free phosphate groups of nucleotides to immobilized iron(III) ions is not based on simple electrostatic interactions, as the chromatography was performed in the presence of 1 *M* sodium chloride. A similar observation was made with a phosphoprotein containing a phosphorylated residue [2]. Phosphorylation of a single serine residue on the histone molecule strengthens its binding to iron(III) chelated gel. Phosphorylated histone may be eluted by an increase in pH. As the chromatography was performed in the presence of 1–4 *M* sodium chloride, non-specific electrostatic interactions could not be the major factor responsible for the increase in pH required for the elution [2].

It should also be pointed out that groups other than phosphate might be able to interact with immobilized trivalent iron. Recent studies have shown that proteins lacking phosphate groups are adsorbed on the gel [3,12]. Our results [4] indicate that in addition to phosphate groups, clusters of carboxylic groups and hydroxy groups on the phenolic ring in tyrosine on proteins are important in the binding to immobilized iron(III) ions. However, among the predictable and documented interactions of immobilized iron(III) ions with proteins the ability to bind phosphate group(s) is most commonly encountered.

This work has demonstrated that for the interaction of nucleotides with immobilized iron(III) ions free, external phosphate groups are required. This observation might be utilized for the separation of mono- and oligonucleotides with exposed phosphate groups from the other constituents of nucleic acids. However, it is not yet clear whether large oligonucleotides can be separated by exploiting the properties of immobilized iron(III) ions. The size limit of nucleotides for effective binding is being investigated.

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