INTERACTIONS BETWEEN HYDROXYAPATITE AND BIOLOGICAL MACROMOLECULES (PROTEINS, NUCLEIC ACIDS)

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Sommaire

On présente un aperçu des données actuellement disponibles sur les groupements chimiques intervenant dans les interactions entre protéines ou acides nucléiques et hydroxyapatite. Ces données dérivent pour l'essentiel de travaux de chromatographie de macromolécules biologiques sur colonnes d'hydroxyapatite.

Abstract

Data available on the chemical groups involved in the interactions between proteins or nucleic acids and hydroxyapatite are presented. Such data essentially derive from investigations on the chromatography of biological macromolecules on hydroxyapatite columns.

Although calcium phosphate was already used by Brücke in 1861 for the purification of pepsin by adsorption and elution and has since found frequent use as a specific adsorbent in protein purification, the modern era in this field only began with investigations carried out in Tiselius' laboratory from 1953 on. In deed, the classical paper of Tiselius, Hjerten and Lewin of 1956 [1], describing the chromatography of proteins on hydroxyapatite has been the starting point for all subsequent investigations. My own interest in hydroxyapatite (HA) goes back to 1958, when I could separate two lipoproteins, α and β lipovitellin, which only differ in their protein phosphate level. For the past 15 years, I have investigated several aspects of chromatography of proteins and nucleis acids on HA. Today, I would like to concentrate on two basic problems in this area, namely 1) the nature of the interaction of HA with nucleic acids and proteins and the chemical groups involved in it: and 2) the effect of secondary structure of biopolymers on their interaction with hydroxyapatite. These and other topics are dealt with in more detail in three recent review articles [2-4].

In the case of nucleic acids it is easy to show that adsorption is due to the interaction of the phosphate groups with calcium on the surface of the HA crystals: 1) *treatment of HA* with compounds having a very strong affinity for Ca^{++}, like EDTA, citrate, and polyphosphates decreases its capacity for nucleic acids; compounds having a lower affinity for Ca^{++}, like phosphates and several carboxylic compounds may be used as eluents; ions having a very low affinity for Ca^{++}, like chlorides, practically do not interfere with adsorption; this is an indication of a *specific competition* by phosphate ions for the Ca^{++} sites. 2) *electrophoresis* of HA crystals in 1 mM phosphate shows that they have a net positive charge. 3) the *sedimentation* rate of HA crystals in 1 mM phosphate is greatly increased by the addition of DNA: this may be due to a decrease in electrostatic repulsion among the positively charged crystals. 4) *phosphoproteins* have a much higher affinity for HA than non-phosphorylated proteins. 5) *chromatography of nucleosides*, some coenzymes and their mono- and polyphosphate derivatives shows that the elution molarity of these substances is only dependent upon their phosphate groups: the non-phosphorylated derivatives are not retained by HA equilibrated with 0.001 M K phosphate (KP); monophosphates are eluted by 0.001 M, but they are retarted; di-, tri, and tetraphosphates are eluted at increasingly higher characteristic phosphate molarities, independently of the organic molecules to which they are bound.

As far as proteins are concerned, the situation can be summarized as follows (see Table).

1) *Acidic polypeptides and proteins* behave similarly to nucleic acids in that their adsorption on HA is little, or not at all, affected by NaCl, KCl or $CaCl_2$. An explanation for this behavior is that the adsorption of acidic polypeptides and proteins, like that of phosphoproteins and nucleic acids, is only, or to a large extent, due to the interaction of their acidic groups with calcium sites at the surface of HA crystals. Elution is,

TABLE

Elution molarities of some proteins from HA columns

Protein	Isoelectric point	Eluting solvents				
		KP 6.8	KP 7.8	KP 5.8	KCl	$CaCl_2$
Lysozyme	10.5	0.12	0.08	0.15	0.25	0.001
Cytochrome c	9.8	0.23	0.20	0.30	0.48	0.007
RNase A	9.7	0.12	0.09	0.15	0.23	0.001
α-Chymotrypsin	8.1	0.16	0.10	0.20	0.32	0.01
Spleen acid DNase	10.2	0.22	0.115	0.32	0.44	0.02
Spleen acid exonuclease		0.125	0.065	0.195	0.25	> 3.0
Myoglobin	7	0.12	0.08	0.17	0.80	> 3.0
Snail acid DNase	5.9	0.11	0.04	0.20	0.54	> 3.0
Pancreatic DNase	4.7	0.04	0.01	0.12	0.4	> 3.0
Bovine serum albumin	4.7	0.06	0.01	0.17	> 3.0	> 3.0
Pepsin	1	0.03	0.01	0.08 0.12	> 3.0	> 3.0

therefore, expected to be caused by anions able to compete with the macromolecule for the calcium sites on HA. It is not surprising that anions having little affinity for calcium, like chloride, are poor eluents compared to the phosphates normally used as eluents. The very high eluting molarities required by phosphoproteins in comparison with nucleic acid may be due, in part at least, to the monoesterified phosphate groups in contrast to the diesterified groups of nucleic acids. Another consideration is that phosphoproteins have runs of phosphorylserines, which form areas of very high density of groups able to interact with HA.

2) The finding that all *basic proteins* can be eluted not only by KP, but also by KCl may be interpreted as indicating that the elution of basic proteins is caused by the cations of the eluents. The fact that the eluting molarities of basic proteins by $CaCl_2$ are 20-200 times lower than those of NaCl or KCl is in keeping with the suggestion that elution is caused by the cations and not by the anions of the eluent. The fact that cations having a very strong affinity for phosphate ions, like Ca^{++} and Mg^{++}, are endowed with a much greater eluting power than cations having a weak affinity for phosphate, like K^+ and Na^+, suggests two important points : a) that the adsorbing sites for basic proteins are to be identified with phosphate groups at the surface of the crystals and b) that elution of basic proteins takes place because of a competition between the cations of the eluent and the basic aminoacid side-groups of proteins for phosphate sites on HA or, in other words, that elution of basic proteins is not simply a ionic strength effect.

It can be noticed that basic proteins are eluted from HA columns at relatively high molarities of phosphate, pH 6.8. The five proteins listed in Table are eluted in the 0.12-0.23 M phosphate range; lysine-rich histone is eluted at en even higher molarity, 0.55 M. In contrast, neutral and acidic proteins seem to be eluted at low phosphate molarities, in the 0.03-0.12 M range (phosphoproteins are an exception to this rule). This different behavior may be understood in the following terms : the usual eluents, NaP or KP, pH 6.8, while very effective, because of their phosphate ions, in competing with the carboxyl groups of proteins for the calcium sites on HA, are much less effective in competing with the basic groups of the proteins for the phosphate sites on HA, because of the low affinity of Na^+ of K^+ of such groups. Therefore, the usual phosphate buffers are good eluents for acidic proteins, as well as for acidic polypeptides, phosphoproteins and nucleic acids, and poor eluents for basic proteins. Conversely, $CaCl_2$ is an excellent eluent for basic proteins and a very poor one for acidic proteins.

An unexpected finding obtained with spleen exonuclease, myoglobin, snail acid DNase and with pancreatic DNase is that these proteins, which are eluted by KCl molarities in the 0.2-0.8 M range cannot be eluted by $CaCl_2$ molarities as high as 3 M. It may be wondered whether in these cases Ca^{++} binds to carboxyl groups of these proteins and strengthens the adsorption by forming bridges to phosphate groups on HA.

The data of Table show that the elution molarity of proteins by KP appears to be increased by a constant factor at pH 5.9 and decreased (also by a constant factor, but with larger fluctuations) at pH 7.8, compared to the usual pH 6.8. Both effects appear to be much greater for acidic than for basic proteins. A satisfactory explanation for this phenomenon is not yet available.

In conclusion, two different types of adsorbing sites exist on HA crystals : calcium sites and phosphate sites. The former appear to bind acidic groups, carboxyls and phosphates; the latter bind basic groups. This picture fits with the known amphoteric character of HA crystals. Elution is caused by anions (usually phosphates), which compete with the carboxyl or phosphate groups of proteins for the calcium sites of HA; or by cations (Na^+, K^+ or, more effectively, Ca^{++} or Mg^{++}), which compete with the basic groups of proteins for the phosphate groups of HA.

From a practical point of view, these results have shown that : 1) elution of proteins from HA columns can be obtained using a number of eluents other than the usual phosphate buffer, pH 6.8; this leads to a remarkable increase in the potentialities of the method; 2) the chromatographic behavior of basic, neutral and acidic proteins on HA columns operated with different elution systems can be predicted to a considerable extent, thus permitting a less empirical approach to separation problems; in turn, the elution molarities of proteins in different solvent systems can be used to identify the nature of the interacting amino-acid side groups.

As far as secondary and tertiary structure effects are concerned, the concept is the following. In contrast with other chromatographic substrates, like substituted cellulose or dextrans, or ion-exchange resins, hydroxyapatite is a crystalline material. Its adsorbing sites are very regularly spaced and find themselves in well-defined positions. This fact automatically implies that the number of possible interactions among these sites and the chemical groups able to interact with them on proteins or nucleic acids will depend upon their distribution on the surface of biopolymers. Changes in this distribution will lead to changes in the number of interaction and therefore in the molarity of eluting ions which can

interfere with such interactions. The recognition that HA can discriminate native and denatured DNA goes back to 1962 [5]. Since that early observation, which has been the starting point for impressive developments in our understanding of the genetic material of eukaryotes, a number of findings have been accumulated confirming that nucleic acids endowed with rigid, ordered structures have more affinity for HA than flexible, disordered ones. As a matter of fact, this rule is a general one and is also valid for proteins. A few examples supporting this idea are the following. Double-stranded nucleic acids, including native and renatured DNA, replicative RNA, and double-stranded synthetic polymers, are all eluted at higher phosphate molarities compared to the corresponding single-stranded ones.

In the case of proteins, the disruption of the secondary and tertiary structures of proteins by 8 M urea or heat causes a strong reduction in their interaction with HA. This phenomenon can be explained by the fact that the random coil configuration of the denatured protein causes a decrease in the number of aminoacid side-groups able to interact per unit of protein surface in contact with HA, and, therefore, a decrease in the elution molarity of denatured proteins. This decrease may be due to the following reasons : 1) acidic or basic groups which were present at the surface of the native protein will, in part, disappear from the "surface" of the denatured protein, which is known to have a random coil configuration; 2) local concentrations (due to the existence of secondary and tertiary structures) of acidic and basic groups will disappear in the denatured state in favor of a more random distribution over all the protein "surface".

It should be very strongly stressed that while the separation of native and denatured biopolymers, particularly double-stranded and single-stranded nucleic acids, has been and is being very widely used, much less has been done to exploit subtler discriminations which HA can achieve within each class. I would like at least to mention separations involving native DNAas having repetitive sequences : the most striking cases concern DNAs having AT-rich spacers like yeast mitochondrial DNAs, the chloroplast and mitochondrial DNA of Euglena. In the case of proteins, very fine separations have been obtained for species showing very small differences, like substitutions at a single amino-acid residue. It is clear that a careful study of the resolving power of the columns in the case of nucleic acids could be very rewarding concerning these separations.

References

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Discussion

G. Rölla : Is it your experience that proteins adsorbed to hydroxyapatite are rapidly denaturated ?

G. Bernardi : No. The contrary is true. Very labile proteins like lipo-proteins can be chromatographed on hydroxyapatite without any problem.

A.S. Posner : Do you have any other proof besides elution to say a carboxyl group of a protein binds to the surface HA calcium ?

G. Bernardi : No.