# Affinity of phosvitin to proteins and polypeptides points to its role in development

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**Investigations directed towards an understanding of the affinity properties of phosvitin, a constituent protein of eggs and oocytes of several species, revealed a hitherto unknown phenomenon of protein/polypeptide binding to this polyanionic protein. Several proteins present in the crude extracts of a variety of tissues, DNA-modifying enzymes, lysozyme, peptide hormones and growth factors exhibited affinity to phosvitin immobilized to a Sepharose matrix. The class of proteins/polypeptides binding to phosvitin is similar to that which binds to heparin, which is known to play a significant role in cellular processes related to growth and function. However, some differences were also observed in the binding of proteins to these two polyanionic macromolecules. Considering the presence of phosvitin in embryonic tissue and its demonstrated affinities for a number of proteins and polypeptide hormones, it would appear that phosvitin plays a significant regulatory role in the developmental events.**

PHOSVITIN is present in large quantities in avian and amphibian eggs. It is formed by the proteolytic processing of the precursor molecule vitellogenin[1]. The synthesis of vitellogenin in liver shows a marked increase in response to estrogen stimulus, which also increases the half-life of vitellogenin mRNA[2]. Vitellogenin synthesized in the liver is secreted into circulation and taken up by the oocyte through a process of receptor-mediated endocytosis[3-5].

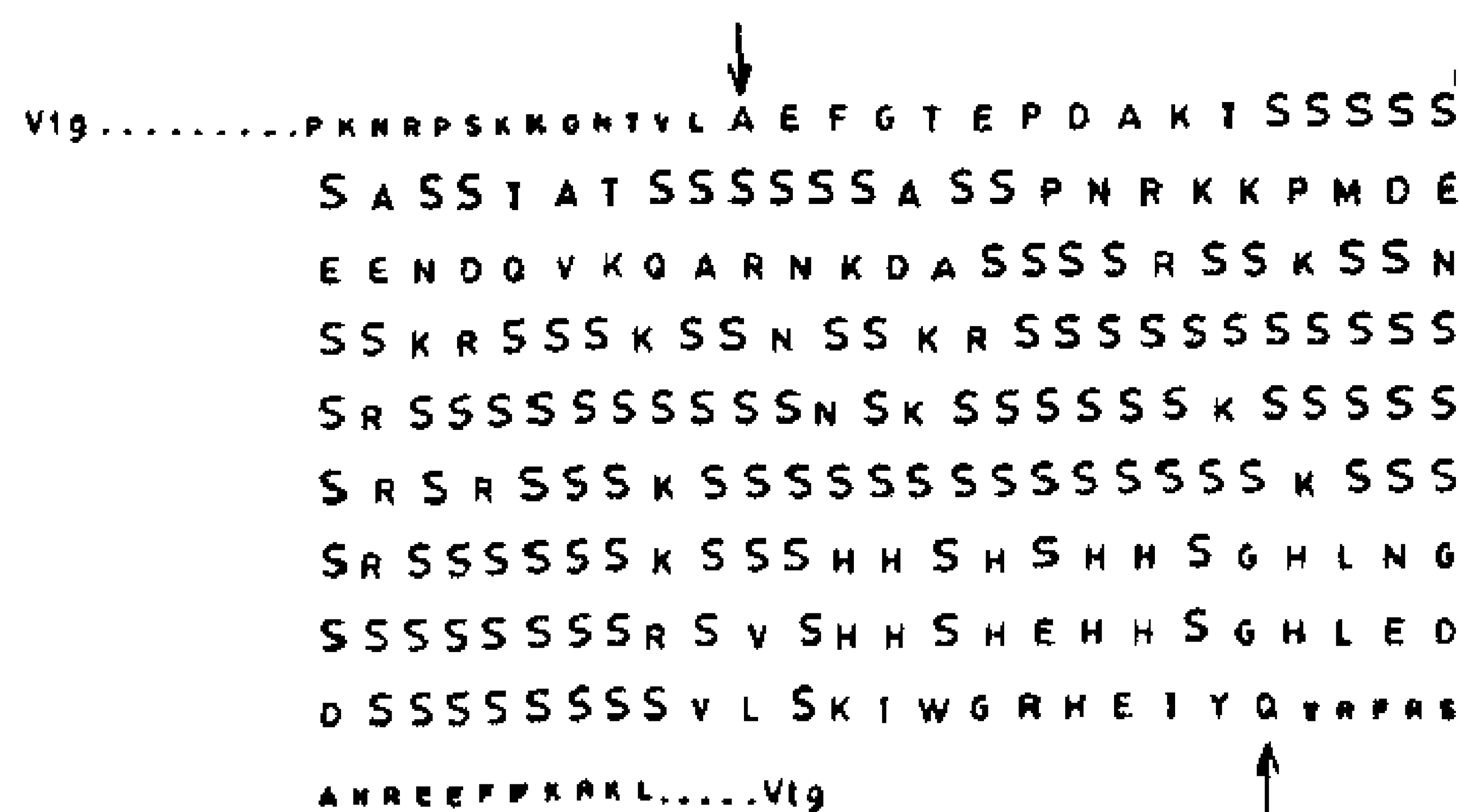


Figure 1. Primary sequence of phosvitin from hen egg yolk

More than 50% of phosvitin is comprised of serine residues, almost all of which are present in the phosphorylated form[6]. The amino acid sequence derived from the cDNA sequence shows serine residues appearing in clusters varying in length from 2–14 residues (Figure 1). The highly polyanionic nature of this protein has led to the belief that it may act as a chelating reservoir for cations such as calcium and iron to meet the developmental needs of the fertilized egg and the early embryo[7]. The binding of cytochrome-c[8] and polylysine[9] to phosvitin and the interaction of a variety of divalent cations with phosvitin[10] has been demonstrated. It is also known that phosvitin can participate in the reversible transfer of phosphate groups with ADP[11]. Although the interaction of phosvitin with a single protein and a polypeptide has been studied, there have been no attempts to investigate the biological significance of such interactions.

In this communication, we describe our attempts to demonstrate the binding of phosvitin to proteins and polypeptides of diverse biological functions. These observations suggest a possible role for phosvitin during the early developmental events.

## Materials and methods

Cytochrome-c and lysozyme were obtained from Sigma, Inc. The restriction enzymes used were purchased from Pharmacia and Bangalore Genie. The synthetic peptide was prepared manually using Boc-protected amino acids.

Phosvitin was purified from hen egg by the procedure of Mecham and Olcott[12]. It was further purified using Mono-Q column on the FPLC system (Pharmacia). The purified phosvitin was coupled to Sepharose using cyanogen-bromide-activated Sepharose (Pharmacia). The amount of protein coupled was determined to be around 6 mg of protein per ml of gel. The phosvitin–Sepharose matrix so prepared was used to demonstrate the affinity of different proteins and polypeptides. Typically, a column (2 ml bed volume) of phosvitin–Sepharose was equilibrated with 20 mM Tris buffer, pH 7.5. The crude extract was applied on the column and excess unbound protein was washed with the same buffer. The bound material was then eluted with a buffer containing NaCl and the fractions were monitored at 280 nm.

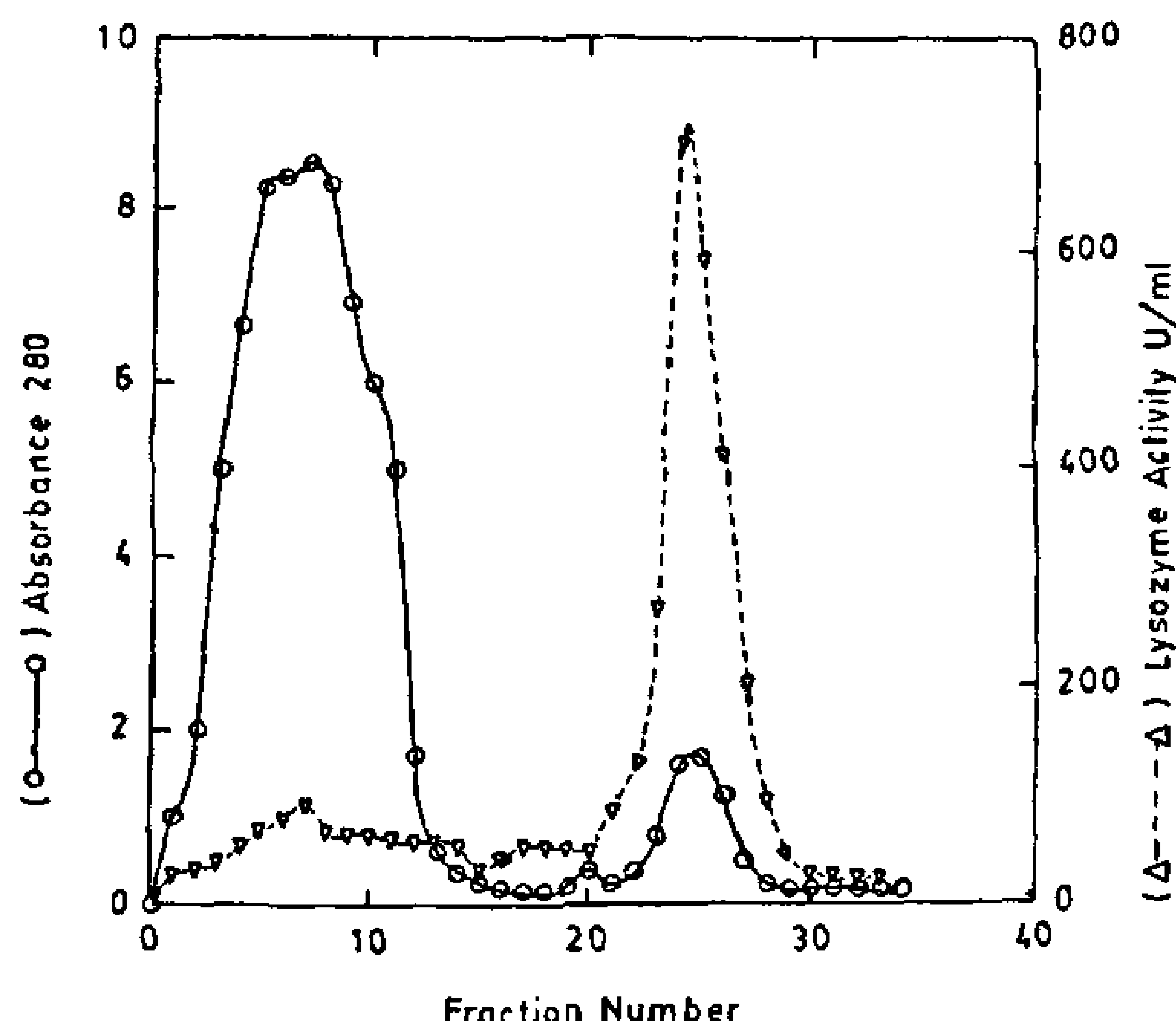



**Figure 2.** Purification of lysozyme from hen egg white on phosvitin–Sepharose. Hen egg white was diluted with 66 mM $KH_2PO_4$ to approximately $A_{280}$ = 10 per ml and 100 $A_{280}$ units were applied to a column (5 ml bed volume). The column was washed to remove unbound protein and eluted with a buffer containing 0.1 M NaCl. The specific activity of the enzyme thus purified by a single affinity step was comparable to the purified commercial sample (specific activity 420.2 units/mg as compared to 437.7 units/mg of the Sigma product).

Assays for the growth hormones were done using the DELFIA kits on the Pharmacia system and the RIA procedures were performed using Diagnostics Inc. kits.

## Results

### *Binding of lysozyme and cytochrome-c to phosvitin*

In our efforts to demonstrate the binding of proteins to phosvitin, we have found that both purified lysozyme and the crude enzymatic activity present in hen egg white bind to a matrix of immobilized phosvitin and can be dissociated by elution with buffer containing salt. In a single step of affinity chromatography, a suitably diluted egg white preparation yielded pure lysozyme comparable in activity to commercially available samples (Figure 2). Under similar conditions, cytochrome-c was also found to bind to phosvitin and was recoverable by dissociation, as described in Table 1.

The analysis of the amino acid sequences of lysozyme and cytochrome-c show that certain basic amino acid residues are present in short stretches and are located either at the carboxy terminal region or at the amino terminal region. It is likely that these domains complement certain phosphoserine clusters in phosvitin. The chemical modification of the lysine and arginine residues involved in the binding of lysozyme and cytochrome-c to phosvitin, and the consequent loss of binding property that was observed in our studies confirm the participation of such charged domains in the interactions (data not shown).

**Table 1.** Affinity of cytochrome-c and lysozyme to phosvitin–Sepharose

| Protein | Phosvitin–Sepharose (mg protein bound/ml gel) |
|---|---|
| 1 Cytochrome-c | 18 |
| 2 Lysozyme | 15 |

To a column of phosvitin–Sepharose (bed volume 1 ml) previously equilibrated with 10 mM Tris–HCl pH 7.5, a solution of cytochrome-c (purified from horse heart, Sigma) was applied, thoroughly washed with the equilibrating buffer to remove the traces of contaminating protein, and eluted with 50 mM sodium phosphate buffer, pH 6.5. Monitoring was done both at 280 nm and 410 nm.

In the case of lysozyme the column was equilibrated with 66 mM potassium phosphate buffer, pH 6.24. Lysozyme, either in the crude form (hen egg white diluted to approximately 10 $OD_{280}$ units per ml) or as purified, was loaded. It was washed to remove unbound protein and then eluted with 66 mM potassium phosphate buffer, pH 6.24 containing 0.1 M NaCl. The recovery was over 90%. Fractions were monitored at 280 nm, as well as for enzyme activity using *M. luteus* cells.

### *Binding of the basic domain peptide of cytochorme-c to phosvitin*

In order to test the participation of a domain region of a protein in the interaction with phosvitin, a synthetic

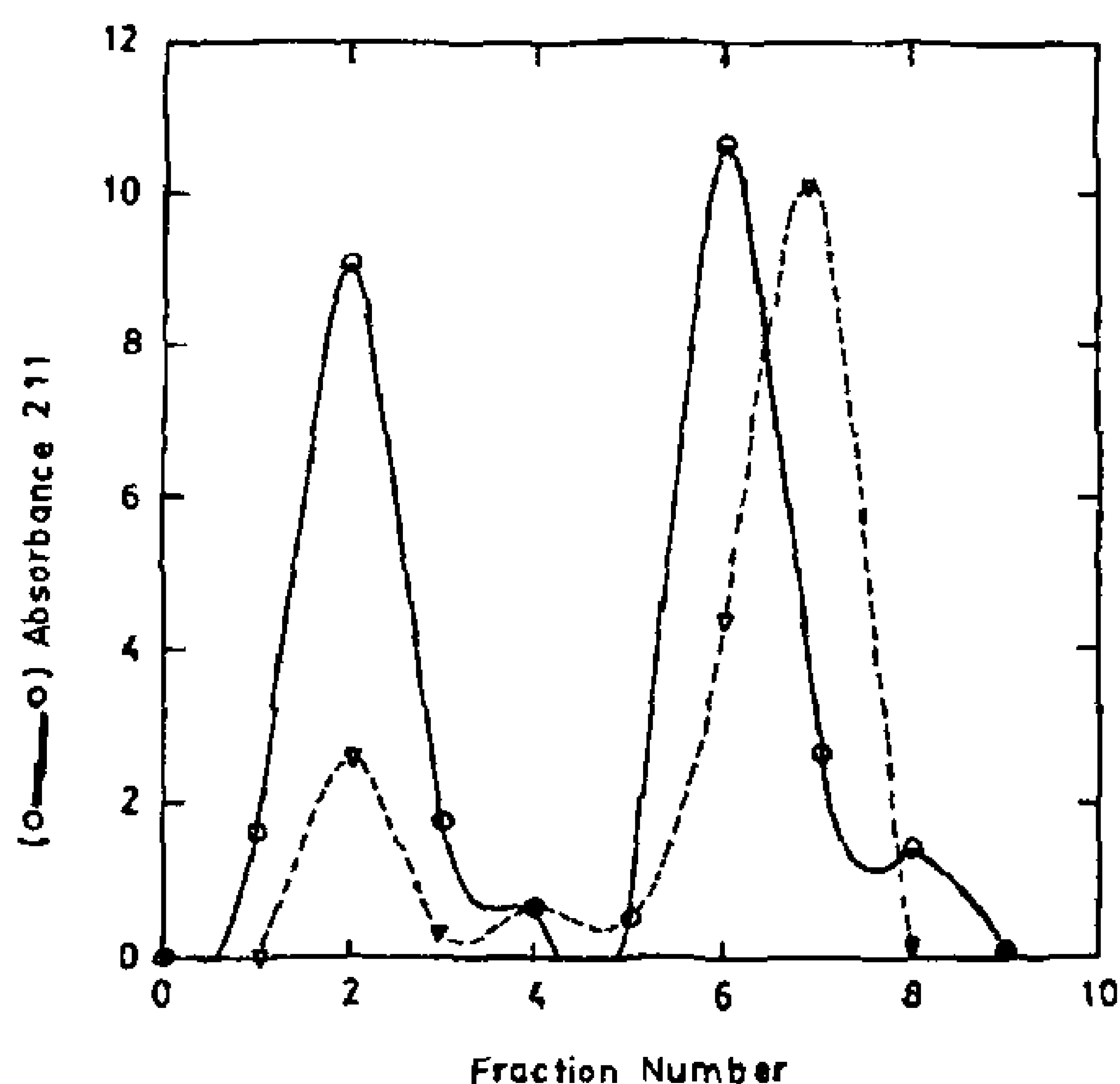
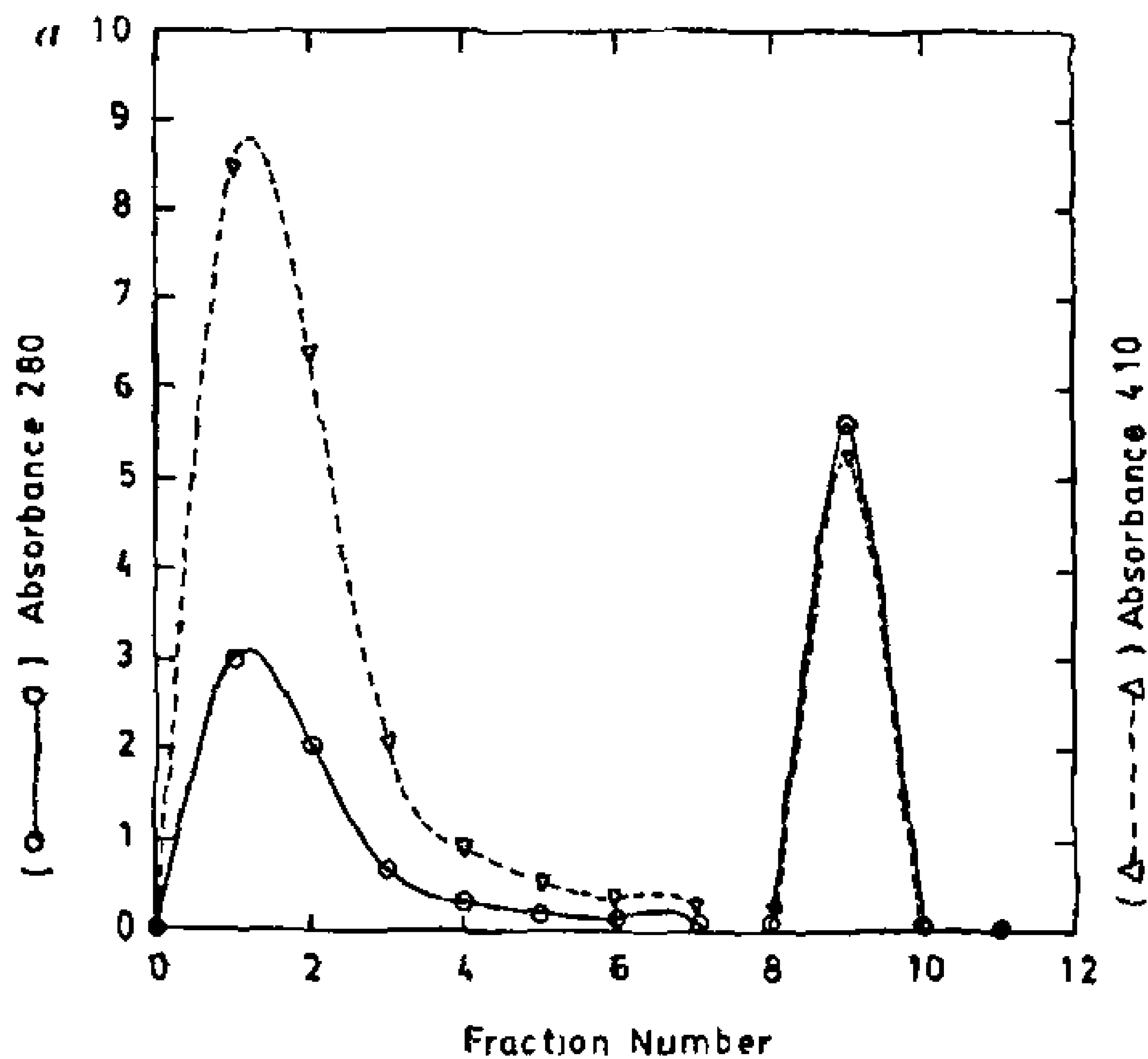



Figure 3. Affinity of carboxy terminal tridecapeptide of cytochrome-c of tuna heart (M I I A G I K K K G E R E) to phosvitin–Sepharose. Phosvitin–Sepharose column (0.2 ml bed volume) was equilibrated with 50 mM Tris–HCl, pH 7.5. The synthetic peptide (2 mg in 2 ml buffer, $A_{211} = 85.95$) was applied and the column washed with the same buffer till $A_{211}$ value reached near zero. The column was first eluted with 1 ml of buffer containing 3 mg cytochrome-c and then washed with buffer alone till both $A_{211}$ and $A_{410}$ were minimum. Finally, the column was eluted with a buffer containing 1.5 M NaCl. The overall recovery was 73%.

tridecapeptide representing a part of the carboxy terminal region of cytochrome-c was employed. As expected, this tridecapeptide was found to bind to phosvitin matrix. Further, the phosvitin–peptide complex thus formed could be dissociated using native cytochrome-c as a competitive ligand (Figure 3) in a displacement chromatographic system.

In the context of such domain interactions invoked, it was thought worthwhile to check whether there were any exclusive domains present in phosvitin for both lysozyme and cytochrome-c. For this purpose, experiments were carried out in which a given quantity of phosvitin Sepharose was saturated with pure lysozyme, following which a solution of cytochrome-c was passed through such a column. In these studies it was found that cytochrome-c was able to displace about 35% of bound lysozyme. In contrast, when cytochrome-c was first bound to saturation on a column of phosvitin- Sepharose and a solution of lysozyme in buffer was next allowed to pass through, there was more than 90% cytochrome-c displacement (Figure 4). Although only 10% of cytochrome-c was not displaceable by lysozyme, it does demonstrate that there are preferential domains available for both proteins on phosvitin.



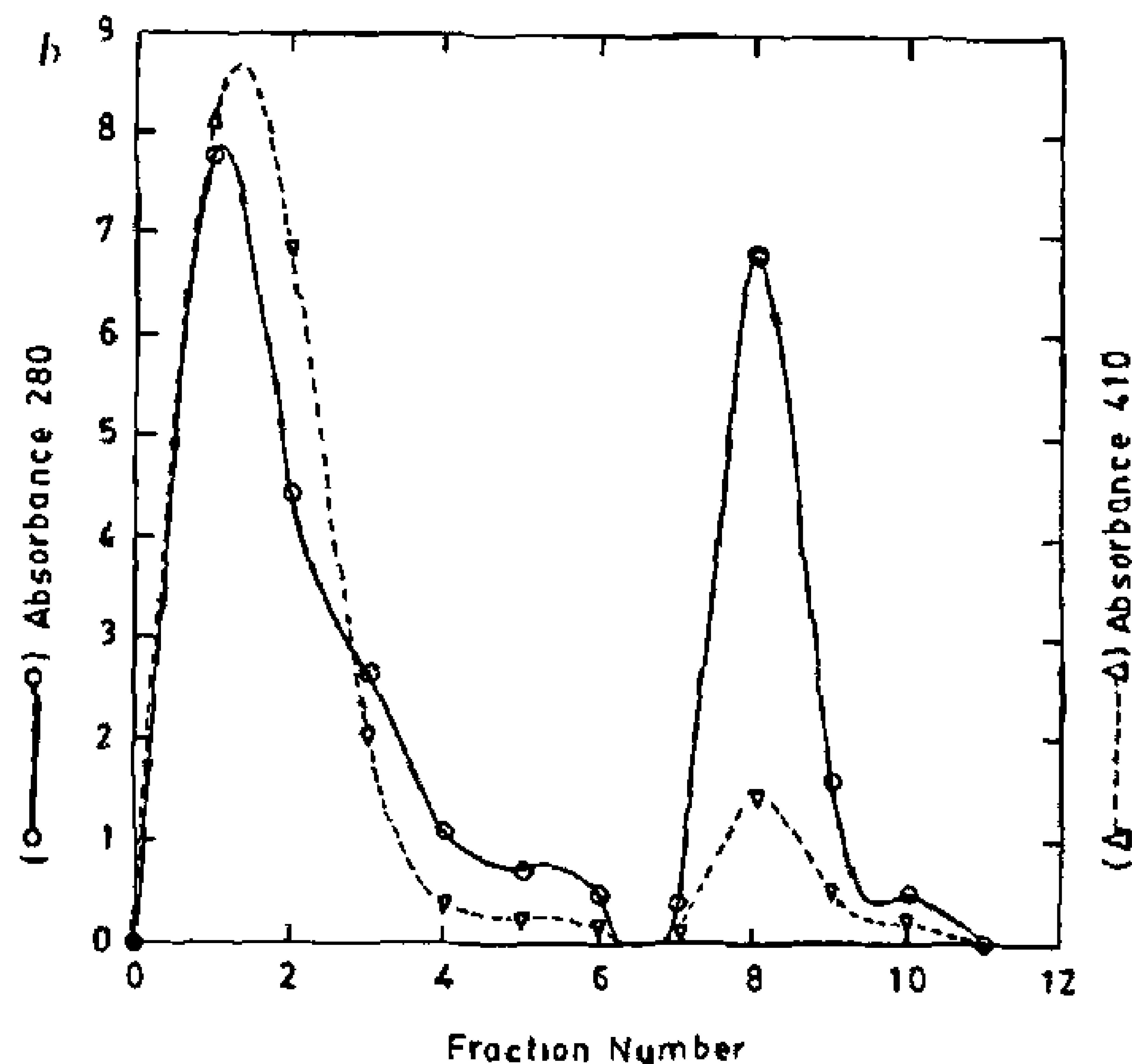




Figure 4. Mutual displacement of phosvitin-bound lysozyme and cytochrome-c. 0.2 ml bed volume of phosvitin–Sepharose columns were equilibrated with 50 mM Tris–HCl, pH 7.5, and were saturated by loading excess of either lysozyme (column A) or cytochrome-c (column B). The columns were washed to remove any unbound protein. Column A was eluted first with cytochrome-c and column B with lysozyme (5 ml, 1 mg protein/ml). The columns were washed again with buffer until the absorbance reached zero. Following this the columns were eluted with a buffer containing 1 M NaCl. Cytochrome-c could displace 35% of the bound lysozyme, whereas lysozyme could displace about 90% of the bound cytochrome-c.

### *Affinity of proteins in crude tissue extracts to phosvitin*

Soluble protein supernatants prepared from homogenates of *E. coli* and from bovine brain and liver were tested for binding to phosvitin. It was observed that a number of proteins present in these extracts exhibit

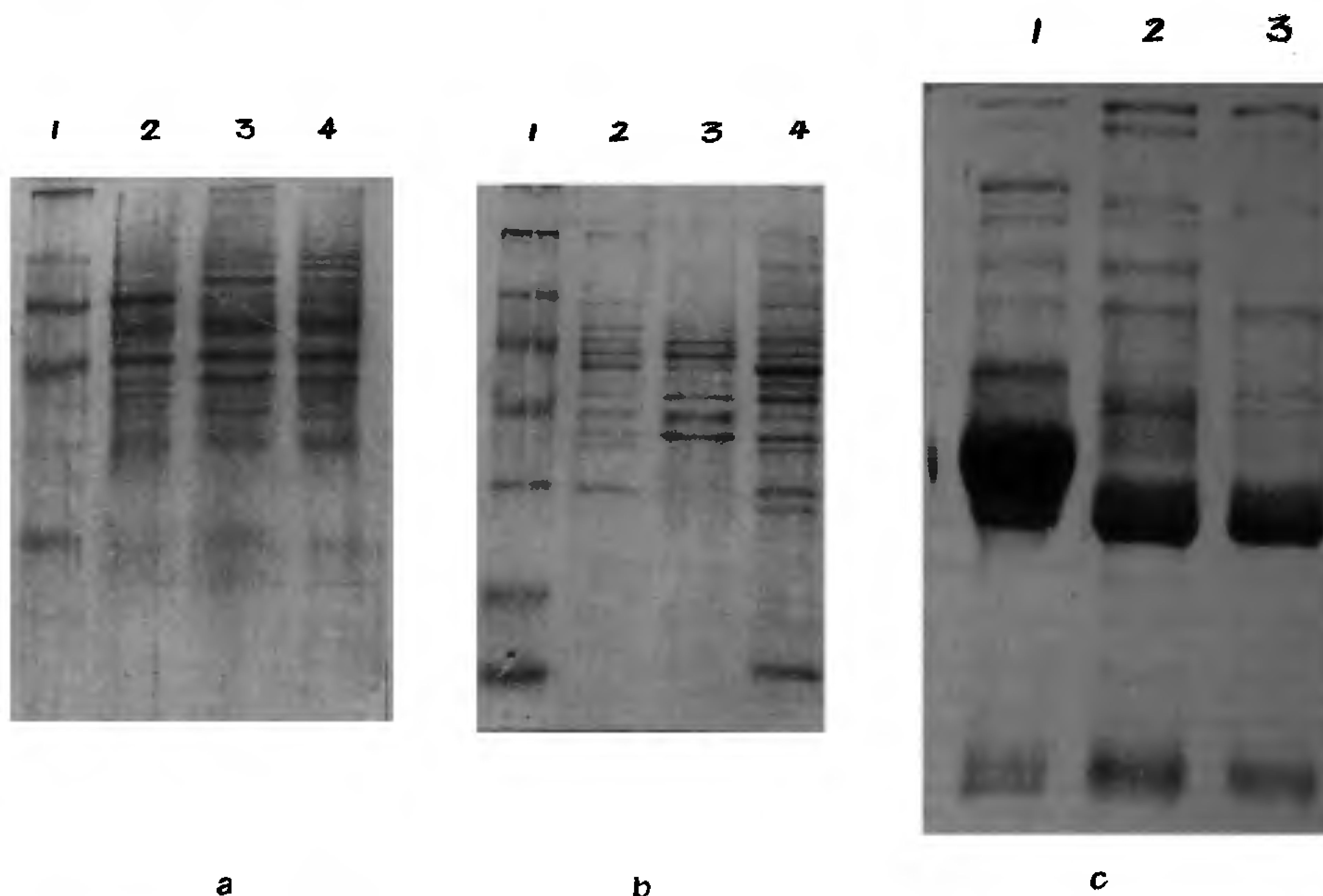



**Figure 5.** Affinity of proteins from tissue extracts to phosvitin–Sepharose. For these experiments phosvitin–Sepharose and heparin–Sepharose 1 ml each were loaded with identical amounts of the indicated tissue extracts. The columns were washed and then eluted with a buffer containing 1.5 M NaCl. The samples, after dialysis and concentration, were analysed by SDS-PAGE and stained with Coomassie blue. *a*, Bovine liver: 1, molecular weight marker, 2, heparin–Sepharose eluate, 3, phosvitin–Sepharose eluate; 4, bovine liver extract. *b*, Bovine brain: 1, molecular weight marker, 2, heparin–Sepharose eluate, 3, phosvitin–Sepharose eluate, 4, bovine brain extract. *c*, Human bone marrow: 1, human bone marrow aspirate, 2, heparin–Sepharose eluate, 3, phosvitin–Sepharose eluate.

**Table 2.** Affinity of DNA-modifying enzymes to phosvitin–Sepharose

| Enzyme | Bound and eluted from phosvitin–Sepharose | Units of enzyme bound* per ml of column |
|---|---|---|
| EcoR I | + | 5000 |
| Hind III | + | 500 |
| Hae III | + | 250 |
| Pst I | + | 300 |
| $T_4$ DNA ligase | + | 800 |

Phosvitin-Sepharose columns of 0.5 ml bed volume were used for evaluation of the affinity of the DNA-modifying proteins. Typically, the column was equilibrated with 10 mM Tris-HCl, pH 7.5, containing 5 mM EDTA and 1 mM DTT (buffer A). The protein was loaded on the column and the column was washed with 15 column volumes of buffer. It was then eluted with buffer A containing 1.5 M NaCl, and dialysed against buffer A at 4°C overnight. Assays were made using lambda DNA, electrophoresed on 0.7% agarose gels, and visualized by ethidium bromide staining.
*Represents 100% binding of enzyme units loaded.

affinity to phosvitin. The liver and brain tissue proteins eluted from phosvitin–Sepharose matrix upon analysis on SDS-PAGE showed several protein bands varying in molecular weights from 15K–120K (Figure 5*a*, *b*). Since heparin, another polyanionic molecule, is also known to bind to several DNA-binding/modifying proteins[13–17], a comparison between phosvitin and heparin was made. The banding pattern of proteins exhibiting affinity for heparin and phosvitin were similar. In the case of some tissue extracts, there were instances of clear differences between proteins bound to heparin and phosvitin. For example, analysis of human bone marrow aspirate chromatographed on heparin and phosvitin shows that some proteins showing affinity towards heparin have no affinity towards phosvitin and some others exhibit very weak affinity to phosvitin (Figure 5*c*).

### *Affinity of DNA-binding/modifying enzymes for phosvitin*

Since the class of proteins present in various extracts binding to phosvitin and heparin appeared somewhat

similar, it was thought worthwhile checking the binding of DNA-modifying enzymes to phosvitin, as they are known to have affinity for heparin. The results (Table 2) suggest that several DNA-modifying enzymes tested possess affinity towards phosvitin.

### *Interaction of peptide hormones and growth factors*

Several polypeptide hormones tested showed affinity to phosvitin (Table 3) It is known that several growth factors, including the basic fibroblast growth factor (bFGF), bind to heparin[18]. Preliminary results also suggest that bFGF exhibits affinity towards phosvitin.

## Discussion

We have shown that several DNA-modifying enzymes and peptide hormones bind to phosvitin. In addition, a number of proteins present in a variety of crude extracts also bind to phosvitin. Thus, phosvitin becomes the first protein to exhibit affinity to a number of proteins, including growth factors. Incidentally, the class of proteins binding to phosvitin appeared similar to the one binding to heparin, a sulphated polysaccharide, as judged by the banding pattern on SDS-PAGE. The comparison of the available amino acid sequences of proteins and polypeptides exhibiting affinity to phosvitin suggests that a core sequence bearing predominantly basic residues may be involved in the observed interaction (Table 4). It is of interest that such sequences have already been implicated in the binding of some of these proteins and growth factors to heparin[19]. For example, by application of recombinant DNA methodologies, a truncated version of bFGF lacking the basic amino acid cluster located near the carboxy terminal has been produced[20]. Interestingly, such a truncated version of bFGF does not bind to heparin[20], suggesting the involvement of a basic domain in bFGF in heparin binding.

Table 3. Affinity of peptide hormones for phosvitin–Sepharose

| Hormone | Assay/detection procedure | Binding and elution |
|---|---|---|
| FSH | DELFIA** | + |
| FSH (Human serum) | DELFIA | – |
| ACTH | RIA† | + |
| ACTH (Human serum) | RIA | + |
| PTH* | RIA | + |
| PTH (Human serum) | RIA | + |
| RCG | DELFIA | N S |
| PROLACTIN | DELFIA | – |
| bFGF (Bovine brain) | SDS-PAGE | + |

*PTH (44–68) mid molecule

**Dissociation enhancement lanthanide fluoroimmuno assay

†Radioimmunoassay

To determine the affinity of growth factors/hormones to phosvitin–Sepharose, typically, columns of 1 ml bed volume were used The column was first equilibrated with 10 mM Tris–HCl, pH 7 75, loaded with protein (either as pure protein or as an extract of the source tissue), washed to remove unbound protein and eluted with 10 mM Tris HCl, pH 7 75, containing 1 5 M NaCl The washed and eluted fractions were assayed for the growth factor/hormones by immunoassay procedures, as indicated

The interaction of proteins with phosvitin in general appeared to be weaker, as evidenced by the dissociation of the complexes by lower concentrations of salt, than what is required to dissociate the heparin–protein complexes. The anionic properties of heparin and phosvitin carrying large domains of sulphate and phosphate groups, while providing the chemical basis for the affinities for proteins and polypeptides observed, can additionally be expected to offer domains of specific structural complementarity to the corresponding basic regions in other macromolecules. Such biophysical considerations should form a topic of considerable interest for further study.

In such a context, from a comparison of the sequence data of certain basic stretches in cytochrome-c (containing one arginine and three lysine residues) and lysozyme (containing two arginine and one lysine), it would appear that the binding of cytochrome-c to phosvitin would be stronger. The results, which are contrary to this expectation, suggest that perhaps the distribution of the charge density in the short stretches as well as the geometry of the domain may be very important for interaction.

The importance of structural component in macromolecular interactions comes from the study of the interactions between LDL and its receptor In a mutant form of LDL where a single amino acid change distal to the site of interaction has been identified was found to have very little affinity to its receptor[21]. In the case of RGD sequences involved in the adhesion phenomenon, a number of other proteins containing RGD sequences fail to function as adhesion molecules[22]. This is directly related to their geometry, which is dictated by immediate neighbours. This again implies the importance of structural complementarity.

It should be emphasized that the structural aspects are important to both the interacting species. Thus, it is increasingly recognized that the many unrelated biological actions of heparin – for example, the acceleration of the rate of thrombin inactivation, which prevents homeostasis, or its action in the regulation of angiogenesis by binding growth factors that enhance their activity and stability, or its ability to inhibit or activate many enzymes – are thought to result from its conformational flexibility.

Such a conformational flexibility of heparin is due to its nonrigid structure arising from the equilibrium between different conformers of α-*l*-iduronate residues[23]. It would be of considerable value to understand the structural properties of phosvitin that render to it affinity properties similar to heparin.

Table 4. Core sequences in proteins/peptides that may be involved in interactions with phosvitin

| | Polypeptide | Possible sequence involved in affinity of the molecule for phosvitin | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Tested for affinity and confirmed | Lysozyme (108–119) | W | V | A | W | R | N | R | C | K | G | I | D |
| | Cytochrome-c (horse heart) | I | F | A | G | I | K | K | K | T | E | R | E |
| | ACTH (12–21) | K | P | V | G | K | K | R | R | R | V | K | V |
| | hPTH (1–34) | R | V | F | W | L | R | K | K | L | Q | D | V |
| | PTH (RP) | S | I | Q | D | L | R | R | R | F | F | L | H |
| | bFGF (103–114) | Y | N | T | Y | R | S | R | K | Y | T | S | W |
| | FSH (38–49) | P | I | P | L | R | S | K | K | T | M | L | V |
| | PTH (44–68) | G | G | S | N | R | P | R | K | K | I | D | N |
| Likely to have affinity based on sequence and affinity to heparin | c-myc | E | D | L | L | R | K | R | R | E | Q | I | H |
| | c-jum | R | I | K | A | E | R | R | K | M | R | N | R |
| | hCG (subunit) | K | E | P | L | R | P | R | C | R | P | I | N |
| | Neurotensin | L | Y | E | N | K | P | R | R | P | Y | I | L |
| | h Apo B100* | R | F | K | H | K | R | R | S | Y | I | Y | |

*In separate studies done on human serum, as described in the text, fractions of human Apo B100 were found to be bound, as determined by clinical laboratory method for LDL analysis

Although extensive conformational studies on phosvitin have been carried out using infrared spectroscopy, circular dichroism[24], NMR[25], Raman spectroscopy[26] and, more recently, vibrational circular dichroism[27], the structural description of phosvitin still remains unclear. It is said to possess a predominantly coil structure at high pH and an antiparallel beta sheet structure at low pH. The NMR results indicate an open and flexible structure at neutral pH, with accessibility of phosphoserines to solvent. There are indications of the presence of parallel sh... turns and other structures, but the X-ray is poorly defined for unequivocal assignment. These results certainly point to the complexity of the molecule. It may also be mentioned here, that attempts to analyse the crystal structure of lipovitellin – phosvitin complex shows no electron-dense regions corresponding to phosphoserine clusters[28]. X-ray-crystallographic studies on phosvitin–protein complexes as well as on model peptides carrying appropriate sequences would be valuable. Studies in this direction are in progress.

In this context, the observed interaction of a number of proteins/polypeptides to phosvitin acquires special significance. Therefore, its presence in embryonic tissue raises some questions regarding its role.

The sequence and regulation of early events in embryogenesis are under extensive investigation by several investigators[29–32]. These studies show that in vertebrate embryos the signal for induction of the mesoderm stems from the endoderm[33]. Several peptide growth factors, including the basic fibroblast growth factor (bFGF), that are inducers of mesoderm in *Xenopus* have been identified[34–36]. Although preformed bFGF is present in the unfertilized egg, it is not clear how the bFGF is sequestered and stored in this tissue until the 64-cell stage in embryogenesis. The observation that exogenously added heparin in between physically separated ectodermal and endodermal explants of *Xenopus* embryo blocked mesoderm induction[37] might be indicative of the possibility of the presence of a molecule similar in chemical nature to heparin that acts as a store for bFGF. The present observations suggest the possibility that naturally present phosvitin, as a part of the lipovitellin complex, may be engaged in a regulatory role by virtue of its affinity to a variety of protein/polypeptide growth factors, in terms of storage by sequestering and delivery by regulated dissociation. Regulation and timing of the release of polypeptides and growth factors from the phosvitin complex may be a consequent function to the gradual dephosphorylation of phosvitin. During embryogenesis, the energy requirement of the embryo is known to increase, resulting in an order of magnitude increase in the ATP–ADP turnover. Should phosvitin be participatory in this process even for a small duration of time, the overall charge characteristics of this protein are bound to be altered, effecting the release of bound proteins. The exact mechanism of protein release from phosvitin is, however, not known. Further probing of the events in embryogenesis in relation to phosvitin might throw light on the functions of this unique protein.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