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The binding requirements of monkey brain lysosomal enzymes to their immobilised receptor protein

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Abstract. The lysosomal enzyme binding protein (receptor protein) isolated from monkey brain was immobilised on Sepharose 4B and used to study the binding of brain lysosomal enzymes. The immobilised protein could bind ß-D-glucosaminidase, α-D-mannosidase, α-L-fucosidase and *β*-D-glucuronidase. The bound enzymes could be eluted either at an acid pH of 4·5 or by mannose 6-phosphate but not by a number of other sugars tested. Binding could be abolished by prior treatment of the lysosomal enzymes with sodium periodate. Alkaline phosphatase treatment of the enzymes did not prevent the binding of the lysosomal enzymes to the column but decreased their affinity, as seen by a shift in their elution profile, when a gradient elution with mannose 6-phosphate was employed. These results suggested that an 'uncovered' phosphate on the carbohydrate moiety of the enzymes was not essential for binding but can enhance the binding affinity.

Keywords. Lysosomal enzyme binding protein; brain; immobilisation; binding requirements.

Introduction

In fibroblasts, lysosomal acid hydrolases are targeted to lysosomes by a membrane bound receptor which recognises mannose 6-phosphate residues on the enzymes (Natowiez et al., 1979; von Figura and Klein, 1979; Ullrich *et al*., 1978; Distler *et al*., 1979). The phosphomannosyl receptor has been purified from bovine liver (Sahagian *et al*., 1981) and from rat chondrosarcoma (Steiner and Rome, 1982). Like the fibroblast receptor these purified receptors also recognise mannose 6-phosphate on the lysosomal enzymes. Synthesis of the phosphomannosyl recognition marker involves the sequential action of two enzymes with the intermediate formation of a phosphodiester (lysosomal enzyme . . . mannose-6-Ⓟ-l-N acetyl glucosamine) (or 'blocked' phosphate) followed by its cleavage to a phosphomonoester (Reitman and Kornfeld, 1981; Hasilik *et al*., 1981; Varki and Kornfeld, 1980; Waheed et al., 1981). Although the mannose 6-phosphate recognition marker has been shown essential for the uptake of lysosomal enzymes by fibroblasts, alternate mechanisms of recognition have been suggested by the following studies:

(i) Studies (Owada and Neufeld, 1982; Waheed *et al*., 1982) on organs obtained from patients with I-cell disease have shown that although the N-acetylglucosamine-1-

Abbreviation used: Con A, Concanavalin A.

phosphate transferase enzyme responsible for the phosphorylation of mannose was absent in this disease, normal or near normal levels of lysosomal enzymes were found in the brain, liver, kidney and spleen.
(ii) Gabel *et al*. (1983) have shown that mutant murine cell lines which lack the mannose 6-phosphate receptor are still capable of sequestering high levels of acid hydrolases in the lysosomes, thereby suggesting the existence of an alternate pathway for the entry of enzymes into the lysosomes. These mutant cells are now demonstrated to have a second type of cation dependent phosphomannosyl receptor (Hoflack and Kornfeld, 1985).
(iii) Talkad and Sly (1983) and Cladaras *et al*. (1983) have shown that the bovine liver receptor could also bind 'blocked' phosphate residues on the lysosomal enzymes.
(iv) Multivalent interactions involving some component of the protein backbone have been suggested to contribute to the enzyme receptor interaction (Karsen *et al*., 1980; Myerowitz and Neufeld, 1981; Rome and Miller, 1980).
(v) Macrophages and liver non-parenchymal cells have been shown to recognise mannose and N-acetylglucosamine residues on lysosomal enzymes (Diment and Dean, 1983; Ullrich *et al*., 1979).
(vi) Freeze (1985) showed that oligosaccharides with two phosphodiesters and the peptide portion of slime mold lysosomal enzymes are important for their recognition by the fibroblast phosphomannosyl receptor.

Hill *et al*. (1985) showed the uptake of *β*-glucosidase from *D. discoideum* and *β*-galactosidase from bovine testes by rat cerebral cortex astrocytes through mannose 6-phosphate receptors. Very little, however, is known about the biosynthesis and packaging of brain lysosomal enzymes in brain cells. We have previously reported the isolation by phosphomannan- Sepharose chromatography of a binding protein (recaptor protein) for lysosomal enzymes from monkey brain (Alvares and Balasubramanian, 1983). The binding of brain lysosomal enzymes by this receptor protein was markedly inhibited by mannose 6-phosphate followed by mannose and N-acetylglucosamine. In this report we describe the interaction of 4 brain lysosomal enzymes with the receptor protein immobilised on Sepharose and demonstrate that the presence of an 'uncovered' phosphate on the carbohydrate moiety of the enzyme is not obligatory for binding to the receptor protein although it may enhance the binding affinity.

Materials and methods

Phenolphthalein glucuronide, *p*-nitrophenyl glycosides, *Escherichia coli* alkaline phosphatase (type III) and the sugars were from Sigma Chemical Co., St. Louis, Missouri, USA, sodium metaperiodate from J. T. Baker and Sepharose 4B from Pharmacia. Concanavalin A (Con A) was prepared from *Canavalia gladiata* and coupled to CNBr-activated Sepharose as described earlier (Surolia *et al*., 1973; Alam and Balasubramanian, 1978). Endoglucosaminidase H (Seikagaku Kogyo Co., Japan) was a kind gift from Dr. K. von Figura, Federal Republic of Germany. All other chemicals were of the highest purity grade available.

Enzyme assays

ß-D-Glucuronidase was assayed using Phenolphthalein glucuronide as substrate (Alvares and Balasubramanian, 1982). N-Acetyl *ß*-D-glucosaminidase, α-D-mannosidase and α-L-fucosidase were assayed using their respective *p*-nitrophenyl glycosides as substrates as described earlier (Alvares and Balasubramanian, 1983). One unit of enzyme activity is defined as 1μmol ofsubstrate hydrolysed in 1 h at 37°C under the standard assay conditions. Purified human serum pseudocholinesterase was prepared and assayed using butyryl thiocholine iodide as substrate (George and Balasubramanian, 1981). Protein was estimated according to Lowry et al. (1951) using crystalline bovine serum albumin as standard.

Isolation of the brain lysosomal enzyme binding protein and its immobilisation

The lysosomal enzyme binding protein was isolated from monkey brain by affinity chromatography on a phosphomannan-Sepharose column (Alvares and Balasubramanian, 1983).

The method of immobilisation involved the activation by CNBr of Sepharose 4B according to the method of March et al. (1974) and the coupling of the receptor protein to the activated Sepharose through a 6 carbon arm (George and Balasubramanian, 1981). The activated Sepharose (2 ml) was suspended in an equal volume of 0·1 M borate buffer pH 9·5 and 0·5 g hexanediamine in 2 ml of the same buffer was added to the gel. The pH was adjusted to 9·5 with 5 M HCl and the gel stirred at 4°C for 24 h. The excess amine was removed by washing the gel with 20 volumes of 1 M NaCl followed by water. The aminated gel was suspended in 2 ml of 20 mM potassium phosphate buffer, pH 5·0 and coupled to 1·3 mg of receptor protein in 2 ml of the same buffer in the presence of l-(3-dimethylaminopropyl)-3-ethyl carbodiimide for 24 h at 4°C. The excess protein was removed by washing the gel with 1 M NaCl. The amount of protein coupled was 0·74 mg.

Chromatography of lysosomal enzymes on the receptor protein -Sepharose column

Con A- Sepharose eluate, rich in lysosomal enzymes, in 20 mM Tris-HCl, pH 7·4 was passed through the receptor- Sepharose column (3 × 0·7 cm) equilibrated with the same buffer, at a flow rate of 5 ml/h. The column was washed with 10 bed volumes of the same buffer and then eluted with either 20 mM citrate-phosphate buffer, pH 4·5 or 20 mM sugars in 20 mM Tris-HCl, pH 7·4. Fractions of 2 ml were collected and assayed for enzyme activity.

In other experiments, the column was eluted with a linear gradient of 0→35 mM mannose 6-phosphate in 20 mM Tris-HCl, pH 7·4 (20 ml in each chamber) at a flow rate of 2 ml/h. This was followed by an elution with 20 mM citrate-phosphate buffer, pH 4·5. Fractions of 1 ml were collected and assayed for enzyme activities.

Periodate treatment

Sodium metaperiodate was added to a final concentration of 10 mM to the enzyme previously dialysed against 20 mM phosphate buffer, pH 6·0. Incubation was carried out in the dark at 4°C for 8 h at which time sodium metaperiodate was once again

added bringing the final concentration to 20 mM and the incubation continued for another 16 h. The reaction was quenched by the addition of 0·2 volumes of 1 M ethylene glycol followed by dialysis against 20 mM Tris-HCl buffer, pH 7·4. Periodate treatment resulted in 10—15% of loss in glucosaminidase, glucuronidase and mannosidase activity and complete loss in fucosidase activity.

Amino acid modifications

Lysine and cysteine residues were modified by trinitrobenzene sulphonic acid according to Fields (1972), tyrosine by N-acetyl imidazole (Riordan *et al*., 1965), arginine by phenylglyoxal (Chang and Huang, 1981) and tryptophan by 2-hydroxy-5-nitrobenzyl bromide (Barman and Koshland, 1967).

Alkaline phosphatase treatment

E. coli alkaline phosphatase digestion was done by incubating 1·25 mg of the Con A-Sepharose eluate with 7·5 units of phosphatase in 1 ml of 40 mM Tris-HCl, pH 7·4 for 1 h at 37°C. The contents were then transferred to a dialysis bag and the incubation continued at 22°C for 2 h while dialysing against 1 litre of 40 mM Tris-HCl, pH 7·4 (Talkad and Sly, 1983). Alkaline phosphatase treatment resulted in a loss of approximately 32%, 20%, 38% and 10% activity for the enzymes glucosaminidase, fucosidase , mannosidase and glucuronidase, respectively.

Purification of ß-glucuronidase and endoglucosaminidase H treatment

ß-Glucuronidase from monkey brain was purified upto the stage of antibody-Sepharose column chromatography (Alvares and Balasubramanian, 1982). To 1 ml (0·65 units) of the purified glucuronidase in 20 mM sodium acetate buffer, pH 5·5 was added 0·1 ml (0·01 unit) of endoglucosaminidase H and incubated at 37°C for 48 h (Lang *et al*., 1984). As control, *ß*-glucuronidase was incubated for the same period without endoglucosaminidase H. There was about 80 % loss in glucuronidase activity during the 48 h incubation.

Determination of bound phosphate in the Con A-Sepharose eluate before and after E. coli alkaline phosphatase treatment

The Con A-Sepharose eluate (0·045 mg protein) was dialysed against water and digested with 0·7 ml of 70 % perchloric acid on a sand bath at 160°C for 2 h followed by the measurement of inorganic phosphate according to Galliard *et al*. (1965).

Results

Binding of lysosomal enzymes to the immobilised receptor protein

Figures 1 and 2 show the binding of brain lysosomal enzymes to the receptor protein-Sepharose column. The column was capable of binding at least 4 different lysosomal enzymes tested, glucosaminidase, fucosidase, glucuronidase and mannosidase. The bound enzymes could be eluted either by mannose 6-phosphate or at an acid pH of 4·5

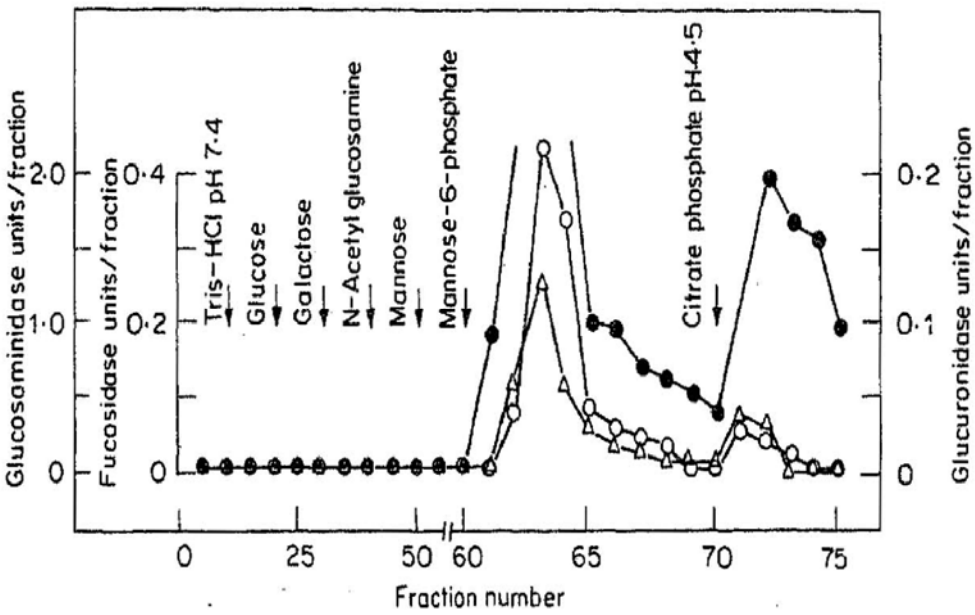

Figure 1. Binding of lysosomal enzymes to the receptor protein-Sepharose column and elution.

Con A-Sepharose eluate containing 68·4 units glucosaminidase (•), 6·8 units fucosidase (o) and 2·32 units glucuronidase (Δ) was loaded on the column and washed as described under 'materials and methods'. Mannosidase binding was not measured in this experiment. There was complete binding of all the enzymes. Arrows indicate the start of elution by 20 mM buffer or different sugars. Recovery of enzyme from the column was 16 % for glucuronidase, 19 % for fucosidase and 38 % for glucosaminidase. Recovery in this experiment was much lower than those following possibly because of the long time taken for elution by various sugars.

(figure 1). The rate of elution with mannose 6-phosphate appeared to be slow and incomplete when compared to the acid pH of 4·5. Glucose, galactose, mannose and N-acetylglucosamine at 20 mM could not elute the bound enzymes (figure 1).

Figure 2 shows the effect of periodate treatment (which results in the cleavage of carbohydrate residues) on the binding capacity of the enzymes. While all the enzymes bound to the column before periodate treatment (figure 2A), after periodate treatment 76 % 100 % and 84 % respectively of glucosaminidase, mannosidase and glucuronidase did not bind and appeared in the breakthrough and wash fractions (figure 2B). Fucosidase lost all the activity on periodate treatment and so could not be monitored for binding.

The requirement of the carbohydrate portion of the lysosomal enzymes for their binding to the immobilised receptor protein was also confirmed using a single lysosomal enzyme namely *ß*-glucuronidase of monkey brain. The purified *ß*-glucuronidase (0·65 units) could completely bind to the column, but after periodate treatment all the enzyme activity remained unbound to the column. An alternate method of carbohydrate removal was done by limited digestion of the purified glucuronidase using endoglucosaminidase H as given under 'materials and methods'. Endoglucosaminidase H is known to cleave the carbohydrate portion from N-asparaginyl linked high mannose glycoproteins. Only limited digestion upto 48 h was possible because even by this period there was 80 % loss in glucuronidase activity. About 10% of the digested glucuronidase remained unbound to the receptor protein-Sepharose column. We verified that only 10% of glucuronidase had been

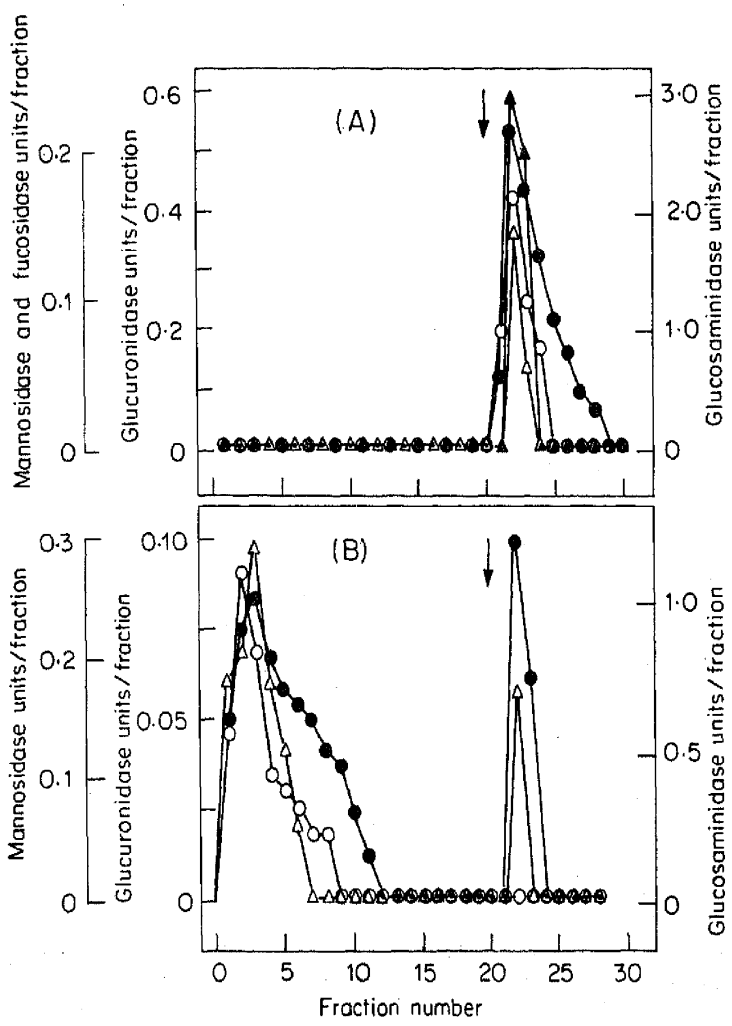

Figure 2. Effect of periodate treatment of lysosomal enzymes on binding to the receptor-Sepharose column.

A. Con A-Sepharose eluate (not treated with periodate) containing 12 units glucosaminidase (•), 0·72 units mannosidase (o), 0·72 units glucuronidase (Δ) and 0·75 units fucosidase (▲) was loaded on the receptor-Sepharose column, washed with 20 mM Tris-HCl pH 7·4 and eluted with citrate-phosphate buffer, pH 4·5. Recoveries of the enzymes were in range of 58—76 %.

B. Periodate treated Con A-Sepharose eluate containing 12·4 units glucosaminidase (•),1·32 units mannosidase (○) and 0·66 units glucuronidase (Δ) was loaded on the receptor-Sepharose column, washed with 20 mM Tris-HCl pH 7·4 and eluted with citrate-phosphate buffer, pH 4·5. Recoveries of the enzymes were in the range of 62-72 %. Fucosidase lost all the activity after periodate treatment and so could not be measured. Arrow indicates start of elution.

deglycosylated by endoglucosammidase H treatment by passing the digested glucuronidase through a Con A-Sepharose column (which binds mannose residues of glycoprotein) whereby the same percentage of enzyme was found to remain unbound to

the column. Untreated glucuronidase completely bound to the same Con A- Sepharose column, while periodate treated glucuronidase remained completely unbound.

Alkaline phosphatase treatment and gradient elution with mannose 6-phosphate

Treatment with E. coli alkaline phosphatase has been used by several investigators to dephosphorylate the mannose 6-phosphate residues on lysosomal enzymes (Ullrich *et al.,* 1978; Diment and Dean, 1983; Talkad and Sly, 1983). Treatment of the brain lysosomal enzymes with alkaline phosphatase did not in any way affect their binding to the receptor- Sepharose column and all the 4 enzymes completely bound as in the phosphatase untreated control experiment. The efficacy of the alkaline phosphatase was checked by measuring the bound phosphate in the Con A-Sepharose eluate before alkaline phosphatase treatment (5·47 µmol P_i per mg protein) and after alkaline phosphatase treatment (0·62 µmol P_i per mg protein) indicating almost 90% removal of the bound phosphate by the phosphatase.

To find out whether phosphatase treatment affects the affinity of the enzymes to the receptor protein, a gradient elution of the enzymes before and after phosphatase treatment by 0 →35 mM mannose 6-phosphate followed by an elution at pH 4·5 was employed. The elution profile of *β*-glucosaminidase under such conditions is shown in

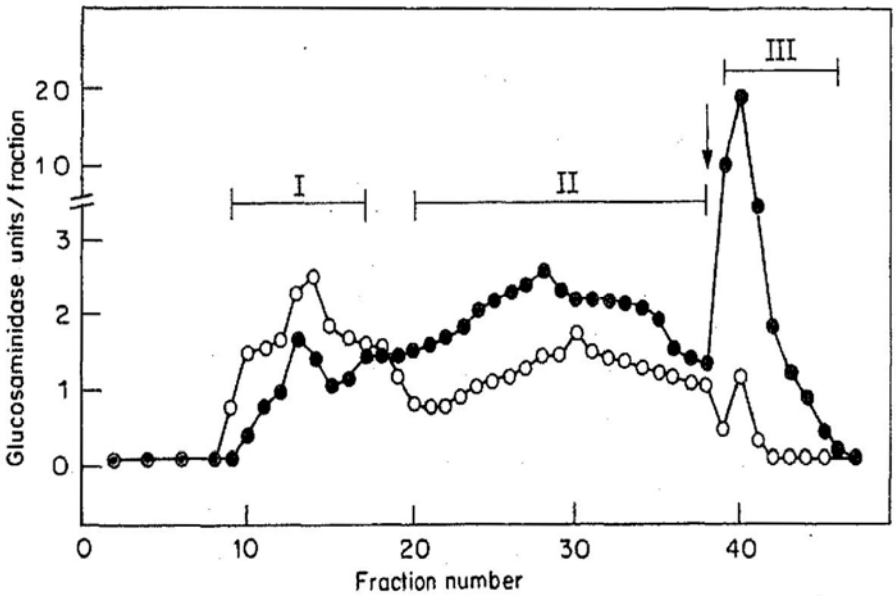

Figure 3. There were 3 peaks of the enzyme both before and after phosphatase treatment

Figure 3. Elution profile of alkaline phosphatase treated (O) and untreated (●) *β* glucosaminidase from the receptor- Sepharose column by a linear gradient (0 →35 mM) of mannose 6-phosphate followed by citrate-phosphate buffer, pH 4·5. Con A-Sepharose eluate containing 150 units of β-glucosaminidase and an alkaline phosphatase treated Con A-Sepharose eluate containing 103 units of *β*-glucosaminidase were subjected to chromatography in separate experiments as given under 'materials and methods'. All the applied enzymes bound to the column completely. Elution with a gradient of mannose 6-phosphate was started from fraction 1 and ended at fraction number 38. Arrow indicates the start of elution with 20 mM citrate-phosphate buffer, pH 4·5. Recovery of enzyme activity from the column was 56 % and 41 % for the untreated and alkaline phosphatase treated enzymes respectively.

as shown in figure 3. Peaks I and II appeared during the mannose 6-phosphate gradient elution and peak III during the subsequent elution with 20 mM citrate-phosphate buffer, pH 4·5. The percentage of glucosaminidase eluted at low concentrations of mannose 6-phosphate (peak I) was only about 7 % ofthe eluted activity before alkaline phosphatase treatment but it increased to 45 % after phospnatase treatment with a significant decrease in peaks II and III (figure 3). These results suggested that a higher

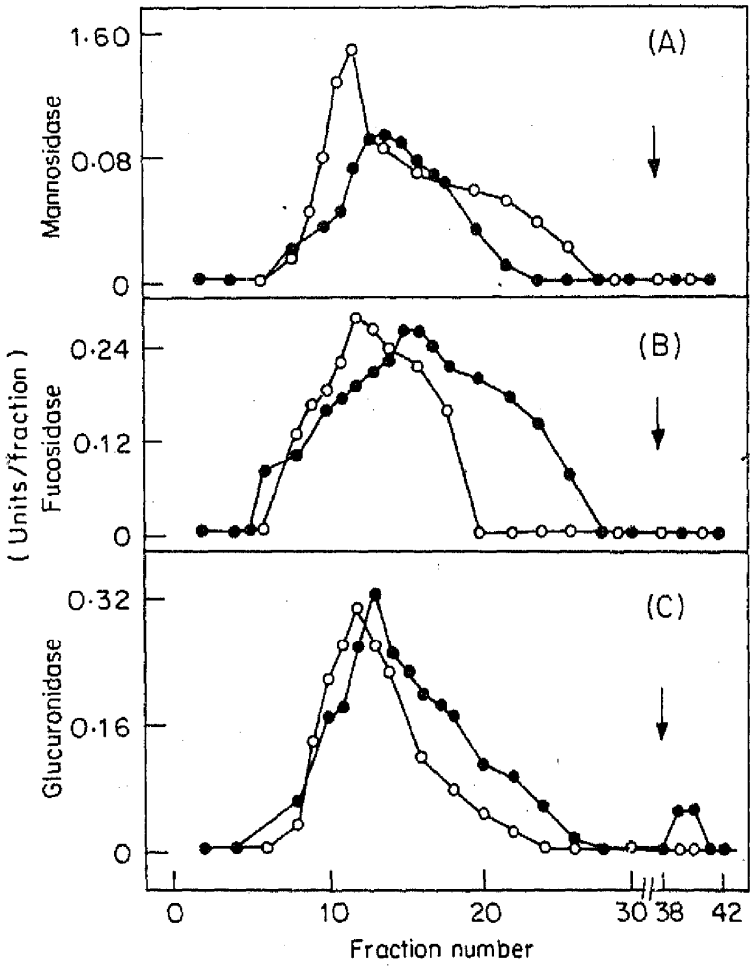

Figure 4. Elution profile of alkaline phosphatase treated (○) and untreated (•) mannosidase (**A**), fucosidase (**B**) and glucuronidase (**C**) from the receptor-Sepharose column by a linear gradient (0 →35 mM) ofmannose 6-phosphate followed by 20 mM citrate-phosphate buffer, pH 4·5.

Con A-Sepharose eluate (containing 5 units of mannosidase, 19 units of fucosidase and 8 units of glucuronidase) and an alkaline phosphatase treated Con A-Sepharose eluate (containing 3·1 units of mannosidase, 15·2 units of fucosidase and 7·2 units of glucuronidase) were subjected to chromatography as given under 'materials and methods'. All the enzymes loaded bound to the column completely. Elution with a gradient of mannose 6-phosphate was started at fraction number 1 and ended at fraction number 38. Arrow indicates the start of elution with 20 mM citrate-phosphate buffer, pH 4·5. Recoveries of enzymes from the column were in the range of 22-30%, 30-35% and 14-20% for the enzymes mannosidase, glucuronidase and fucosidase, respectively.

concentration of mannose 6-phosphate and acid pH of4·5 was needed for the elution of the major percentage of phosphatase untreated glucosaminidase whereas lower concentrations of mannose 6-phosphate could elute the major proportion of phosphatase treated glucosaminidase.

Figure 4 shows the elution profiles of mannosidase, fucosidase and glucuronidase during the gradient elution. These enzymes unlike the glucosaminidase emerged as single peaks. Prior treatment with alkaline phosphatase resulted in a shift of the elution peaks of all the 3 enzymes towards lower concentrations of mannose 6-phosphate. There was also less trailing of the enzymes after phosphatase treatment (figure 4).

Inability of serum pseudocholinesterase to bind to the receptor-Sepharose column

Serum pseudocholinesterase is a non-lysosomal glycoprotein capable of binding to Con A (George and Balasubramanian, 1981). When this enzyme was passed through the receptor-Sepharose column almost 92% of it was recovered unbound to the column and there was no detectable activity on elution of the column at pH 4·5.

Modifications of amino acids

Modifications of the amino acids residues of brain lysosomal enzymes by treatment with trinitro benzene sulphonic acid (for lysine, cysteine), phenylglyoxal (for arginine), N-acetyl imidazole (for tyrosine) and 2-hydroxy-5-nitro benzyl bromide (for tryptophan) as described under methods did not affect their binding to the receptor protein- Sepharose column (data not presented).

Discussion

It is possible that the lysosomal enzymes prepared from monkey brain used in the present studies is a mixture of phosphorylated and dephosphorylated forms (dephosphorylation of the mannose 6-phosphate residue occurs within the lysosomes) and also those containing phosphodiester residues. The almost complete abolition of binding to the receptor protein-Sepharose column by periodate treatment of the lysosomal enzymes clearly suggests that carbohydrate residues are essential for their binding. But the inability of phosphatase treatment to abolish or reduce the binding of any of the lysosomal enzymes to the column suggests that the phosphate moiety in mannose 6-phosphate is not absolutely essential for binding of the enzymes and that the brain receptor protein may recognise mannose or 'blocked' phosphate residues. These results are similar to the recent findings of Freeze (1985) on the interaction of alkaline phosphatase treated D. discoideum lysosomal enzymes with the immobilised phosphornannosyl receptor. This is also reflected in the ability of mannose and N-acetylglucosamine to inhibit the binding of the lysosomal enzymes to the soluble brain receptor reported earlier (Alvares and Balasubramanian, 1983). The brain receptor therefore appears analogous to the receptors in macrophages (Diment and Dean, 1983) and liver nonparenchymal cells (Ullrich et al., 1979) which recognise mannose and N-acetylglucosamine. It is also quite possible that more than one type of receptor is involved in the binding of the enzymes as shown in macrophages (Shepherd et al., 1984). These observations are also relevant to the findings of normal levels of lysosomal

enzymes in the brain and other organs of patients suffering from I-cell disease (Waheed et al., 1982; Owada and Neufeld, 1982).

It is not clear why mannose and N-acetylglucosamine while inhibiting binding to the receptor protein (Alvares and Balasubramanian, 1983) failed to release the enzymes from the receptor- Sepharose column. One possibility is that even though the brain receptor can recognise mannose or N-acetylglucosamine in the absence of mannose 6-phosphate, the latter is still the better recognition marker and the one which is the 'best fit' to the binding site and so the only one potent enough to reverse the binding. Another possibility is that mannose and N-acetylglucosamine alone have too low an affinity to the receptor to be able to reverse the binding, but due to the large amounts of mannose and/or 'blocking' N-acetylglucosamine present on the enzyme molecule the residues interact co-operatively to provide the multivalent ligands with an affinity great enough to bind to the receptor (Talkad and Sly, 1983).

Results on the elution pattern of the lysosomal enzymes before and after phosphatase treatment from the receptor column by a gradient of mannose 6-phosphate show that after phosphatase treatment the major proportion of the enzymes could be eluted at lower concentrations of mannose 6-phosphate. This is more apparent in the case of β-glucosaminidase (figure 3). It is presumable that peak I of glucosaminidase is a dephosphorylated or less phosphorylated form of enzyme as compared to peaks II and III. These results lend support to the suggestion that although removal of phosphate from the lysosomal enzymes does not prevent their binding to the receptor column, it decreases the affinity with which they bind to the column. Although an 'uncovered' phosphate is not essential for binding the presence of phosphate is able to enhance the binding affinity.

The experiment showing that pseudocholinesterase, a non-lysosomal glycoprotein which binds to Con A does not bind to the receptor-Sepharose column rules out the possibility that the brain receptor used in this study behaves as a lectin that recognises mannose residues on glycoproteins in general. Conversely it strengthens the suggestion that the receptor isolated from brain is specific for lysosomal enzymes and can bind lysosomal enzymes through mannose 6-phosphate, mannose or 'blocked' phosphate residues although the mannose 6-phosphate recognition marker appears to be the one with higher affinity.

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