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A BINDING PROTEIN FOR LYSOSOMAL ENZYMES ISOLATED FROM BRAIN BY PHOSPHOMANNAN-SEPHAROSE CHROMATOGRAPHY

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SUMMARY. We have isolated from monkey (Macaca radiata) brain lysosomal fraction by phosphomannan-Sepharose chromatography a protein that binds four different lysosomal enzymes, β-hexosaminidase, β-glucuronidase, α-L-fucosidase and arylsulfatase. The isolated protein which appeared in an aggregated homogeneous form on gel electrophoresis under non-denaturing conditions at both pH 8.3 and pH 5.0 was found to be heterogeneous on SDS-gel electrophoresis with molecular weights less than 67,000. Binding was partly abolished by periodate treatment or by alkaline phosphatase treatment of the lysosomal enzymes. Binding was completely abolished by pronase digestion of the binding protein. Of the different sugars tested for inhibition of binding, mannose-6-phosphate was most effective followed by mannose and N-acetyl glucosamine while glucose and fucose were ineffective.

INTRODUCTION. Since the first proposal of the existence of cell surface receptors for lysosomal enzymes (1), several lines of evidence arising from enzyme internalization studies and binding experiments using intact fibroblasts or isolated membranes have indicated their presence in fibroblasts and rat liver (2-8). It has been shown in fibroblasts that a terminal mannose-6-phosphate residue present on the lysosomal enzymes is the recognition marker for receptors (9,10). However, recent studies (11,12) on organs obtained from patients with I-cell disease have shown that although the enzyme UDP-N-acetyl glucosamine lysosomal enzyme N-acetyl glucosamine 1-phosphate transferase responsible for the synthesis of the mannose-6-phosphate recognition marker was absent, normal or near normal levels of lysosomal enzymes were found in the brain, kidney, spleen and liver of these patients.

Compared to the wealth of information on the processing of lysosomal enzymes, studies on the receptors of lysosomal

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enzymes are limited. A receptor from bovine liver that binds β-galactosidase (13) and from rat chondrosarcoma that binds α-iduronidase (14) have been purified. Both these receptors appeared specific for the mannose-6-phosphate recognition marker as evidenced by the reversal of binding by mannose-6-phosphate. We report here the isolation of a protein from monkey brain lysosomal fraction that binds at least four lysosomal enzymes and the effect of sugars on binding.

MATERIALS AND METHODS. Phenolphthalein glucuronide, p-nitrophenyl phosphate, p-nitrophenyl glycosides, the sugars, pronase P and marker proteins were from Sigma Chemical Co. Nitrocatechol sulfate was prepared as described earlier (15). Phosphomannan was a kind gift from Prof. C.E. Ballou, University of California, Berkeley, U.S.A.

Enzyme assay. β-Glucuronidase was assayed using phenolphthalein glucuronide as substrate (16). N-Acetyl β-D glucosaminidase, α-D mannosidase, α-L fucosidase were assayed using the respective para nitro phenyl glycoside, alkaline phosphatase using p-nitrophenyl phosphate and aryl sulfatase using nitrocatechol sulfate as substrate as described earlier (17). One unit of enzyme activity is defined as 1 μmole of substrate hydrolysed in 1h under the standard assay conditions.

Preparation of phospomannan-Sepharose affinity column. The phosphomono ester was prepared by heating the phosphodiester form of phosphomannan in 0.1N HCl for 30 min at 100°C followed by dialysis against water and freeze drying (18). 10 ml of Sepharose 6-B (Pharmacia) was activated with 1, 4- butanediol diglycidyl ether according to Porath (19). The phosphomonoester (50 mg) was then stirred with the activated gel at pH 12.5 and 50°C for 10h (19). The gel was extensively washed with water and 1M NaCl at pH 7 before use.

Solubilization and isolation of the binding protein. All procedures were done at 0-4°C unless otherwise stated. A 10% (W/V) homogenate of fresh monkey (Macaca radiata) brain was prepared in 0.32M sucrose/ 1mM EDTA using Potter Elvehjem homogeniser. The homogenate was fractionated according to the method of Eichberg et al (20). The P_2 fraction enriched in lysosomes was suspended in 20 mM Tris HCl pH 7.4 and stored at -20°C until used. To the thawed suspension Triton X-100 (0.2% v/v) was added, stirred for 30 min and centrifuged at 67,000 g for 60 min. The residue was collected, extracted with 0.5% (v/v) Triton X-100 in 20mM Tris HCl pH 7.4 and centrifuged as above. The residue after the 0.5% Triton extraction was suspended in 2% Triton X-100 in 20mM Tris HCl pH 7.4 stirred overnight and centrifuged at 67,000 g for 60 min. The supernatant was used for preparation of the binding protein. The supernatant was diluted 10 times with 20mM Tris HCl pH 7.4 and passed through the phosphomannan-Sepharose column previously equilibrated with 20mM Tris HCl buffer pH 7.4/0.2% Triton X-100, The column was washed extensively with the equilibration buffer and then eluted with either 20mM citrate phosphate buffer pH 4.5/0.2% Triton X-100 or 100mM mannose in 20mM Tris HCl pH 7.4/0.2% Triton X-100. The eluate was then dialysed extensively against 20mM Tris HCl pH 7.4, concentrated over aquacide II (CalBiochem) and once again dialysed before use.

Lysosomal enzymes. An $(NH_4)_2 SO_4$ fraction prepared from frozen monkey brain was subjected to Concanavalin A-Sepharose chromatography and eluted with α-methyl glucoside as described earlier (16). The eluate was further concentrated to about 5 times and used as the source of lysosomal enzymes. The enzyme solution was brought to 25% saturation of ammonium sulfate stirred for 30 min and then centrifuged at 12,000 g for 45 min to remove any protein/enzyme that was precipitable. The supernatant was then dialysed exhaustively against 20mM Tris HCl pH 7.4 to remove ammonium sulfate.

Binding assay. Prior to assay for binding activity, Triton X-100 was removed by precipitation of the protein with 4 vol. of acetone previously cooled to -20°C, followed by centrifugation at 12,000 g for 30 min at 4°C. The binding assay used in this study was a modification of the method used for the asialo-glycoprotein receptor (21). Binding mixtures contained in a final volume of 0.6 ml, a concentrated fraction of lysosomal enzymes, 40 mM Tris HCl pH 7.4 and the Triton free binding protein. After incubation at 4°C for 60 min, the enzyme-binding protein complex was precipitated by the addition of 0.2 ml of cold saturated ammonium sulfate adjusted to pH 7.4. After standing for 30 min at 4°C the suspension was centrifuged at 12,000 g for 45 min. The pellet was washed with 25% ammonium sulfate in 20mM Tris HCl pH 7.4, suspended in 0.2 ml of the same buffer and assayed for bound enzyme. Control tubes containing buffer instead of binding protein were treated identically right from the acetone precipitation step.

Periodate treatment of the lysosomal enzymes for 2 to 12 h was done as described earlier (16). Alkaline phosphatase treatment was carried out by incubating 1 ml of the enzyme with 2 units of E.coli alkaline phosphatase in 20 mM Tris HCl pH 8.0/ 0.1M $MgCl_2$ for 2 h at 37°C (16). Pronase treatment of the binding protein was carried out by incubating 30 ug of the protein with 2mg of pronase in 20 mM Tris HCl buffer pH 7.5 for 4h at 37°C. The reaction was stopped by immersing the tube in a boiling water bath for 1 min. In the control tube pronase was added just before heating.

Polyacrylamide gel electrophoresis was performed on 7% acrylamide gels according to Davis (22) in either 0,05M glycine-Tris buffer pH 8.3 or β-alanine-acetic acid buffer pH 5 at 2mA tube till the tracking dye just emerged. Gels were stained for protein by Coomassie Brilliant Blue R. Enzyme activity was detected by cutting the gels into 1mm slices followed by elution of each slice by 20mM phosphate buffer pH 7.0/0.1% Triton and assay. SDS polyacrylamide gel electrophoresis was done in 10% gels containing 0.1% SDS according to Laemmli (23) using the marker proteins γ-globulin (heavy chain 50,000, light chain 23,500), bovine serum albumin (67,000), ovalbumin (45,000) and myoglobin (17,800). Protein was measured using crystalline bovine serum albumin as standard (24).

RESULTS. The lysosome enriched fraction was sequentially extracted with 0.2% and 0.5% Triton to ensure the complete removal of all endogenous lysosomal enzymes. Thus, the 2% Triton supernatant which was used to prepare the binding protein was free of any endogenous glycosidase activity.

Gel Electrophoresis: Figure 1 shows the polyacrylamide gel electrophoretic pattern of the binding protein at pH 8.3 and

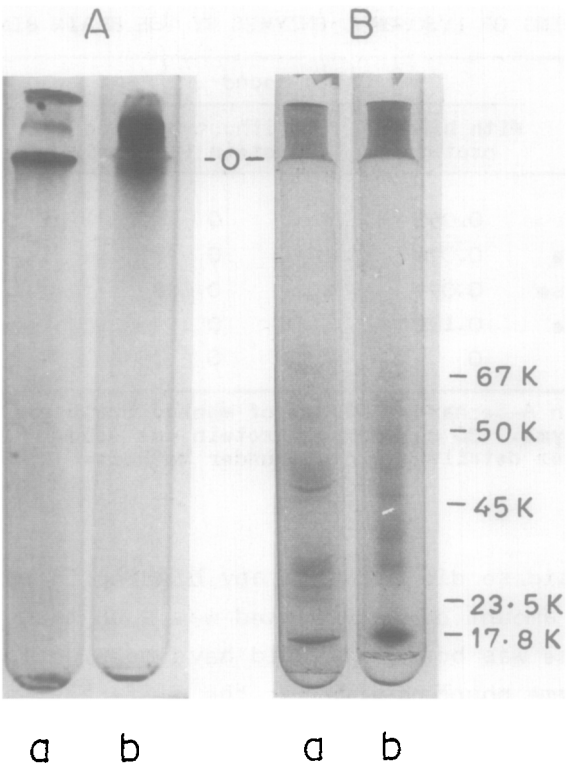

Fig 1. **A. Polyacrylamide gel electrophoresis of 20 µg of acetone precipitated purified binding protein, (a) at pH 8.3 using glycine-Tris buffer and (b) at pH 5.0 using alanine-acetic acid buffer. O stands for origin. B. SDS-gel electrophoresis of acetone precipitated purified binding protein after heating in the presence of 1% SDS and 5% 2-mercaptoethanol. The protein eluted with (a) 20 mM citrate-phosphate pH 4.5 or (b) 100 mM mannose from the phosphomannan-Sepharose affinity column was used. Full details are given under methods.**

pH 5.0. In both cases the binding protein stayed in the spacer gel indicating that it was probably a highly aggregated protein. There were no other protein bands in the running gel. SDS gel electrophoresis resulted in a major band corresponding to a molecular weight of 17,800 and several minor bands with molecular weights less than 67,000. It is presumed that the different protein bands in SDS gel electrophoresis arise from deaggregation of the native aggregated binding protein. A similar observation has been made (21) with the purified asialoglycoprotein binding receptor of rabbit liver.

Specificity of the binding protein: Five lysosomal hydrolases, glucuronidase, hexosaminidase, fucosidase, mannosidase and arylsulfatase were checked for binding to the protein. With the exception of mannosidase all the other four enzymes were bound

TABLE I. BINDING OF LYSOSOMAL ENZYMES BY THE BRAIN BINDING PROTEIN

Enzyme	Units bound		Percentage of total activity bound
	With binding protein	Without binding protein (control)	
α-L-fucosidase	0.058	0	72
β-glucuronidase	0.026	0	62
β-hexosaminidase	0.092	0.008	10
aryl sulfatase	0.192	0	8
α-mannosidase	0	0	0

A Concanavalin A-Sepharose eluate of monkey brain was used as the source of enzyme. 30 μg binding protein was used. Binding assay, units and other details are given under methods.

(Table 1). Mannosidase did not show any binding in repeated experiments. The amount of enzyme used was such that even if 0.5% of mannosidase was bound it would have been detected. The percentage of enzyme bound varied for the different enzymes and were in the order of fucosidase > glucuronidase > hexosaminidase > arylsulfatase (Table 1). Alkaline phosphatase, a non-lysosomal glycoprotein enzyme present in the Concanavalin A eluate (25) did not bind. Furthermore when the affinity chromatography was done in a Sepharose column prepared using 1,4 butanediol diglycidyl ether without phosphomannan under identical conditions, the resulting eluate did not contain any binding protein suggesting an affinity of the binding protein to the phosphomannan ligand.

General properties of binding protein. Binding was saturable for all the enzymes with increasing enzyme concentration. The binding of all the enzymes to the protein increased linearly with increasing concentrations of the receptor protein upto 200 μg. Binding was pH dependent showing a maximum binding at pH 7.5 for all the enzymes with an appreciable decrease of 50% (glucuronidase) to 78% (fucosidase) in binding at pH 4.5.

Treatment of the binding protein with pronase for 4h as described under methods, led to a complete abolition of binding to all enzymes, establishing its protein nature. On the other hand, heating the protein at 100°C for 5 min resulted in only 35-50% loss in binding to the enzymes.

Binding of lysosomal enzymes to the protein was confirmed by gel electrophoresis. The ammonium sulfate pellet containing the

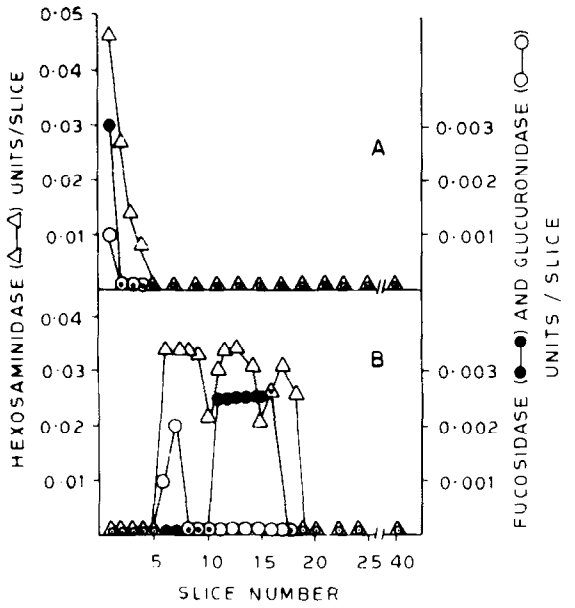

Fig 2. Activity profiles of hexosaminidase (Δ), glucuronidase (O) and fucosidase (●) in the gel slices after electrophoresis. (A) Enzyme-binding protein complex. (B) Free enzyme. Slice number 1 is spacer gel.

precipitated enzyme-binding protein complex was exhaustively dialysed to remove $(NH_4)_2SO_4$ and then electrophoresed using glycine-Tris buffer pH 8.3, as given under methods. In the case of the protein-enzyme complex all the enzyme activities were found in the spacer gel (Fig.2A). On the other hand, the free enzymes (Fig.2B) had a greater electrophoretic mobility with no activity present in the spacer gel.

Carbohydrate specificity of binding protein. Treatment of the enzymes with periodate for 6h resulted in a 17% and 39% loss in binding for glucuronidase and hexosaminidase respectively. Treatment for 12h resulted in a 37% loss in binding of glucuronidase and complete abolition of the binding of hexosaminidase. Effect of periodate on fucosidase could not be studied as the enzyme lost 90% activity even at 2h of treatment. Treatment of the lysosomal enzymes with alkaline phosphatase resulted in a loss of binding of 17%, 35% and 33% for glucuronidase, hexosaminidase and fucosidase respectively.

Fig 3 shows the inhibition of binding by mannose 6-phosphate mannose and N-acetyl glucosamine. Although mannose 6-phosphate was the most potent inhibitor, mannose and N-acetylglucosamine were also able to inhibit the binding of all the enzymes to a

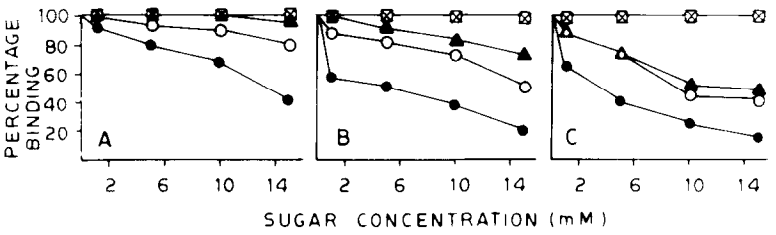

Fig 3. Inhibition of binding of hexosaminidase (A), fucosidase (B) and glucuronidase (C) by mannose 6-phosphate (●), mannose (○), N-Acetylglucosamine (▲), glucose (×) and fucose (□).

lesser extent. In the case of glucuronidase both mannose and N-acetylglucosamine were equally effective in inhibition of binding. Two other sugars, fucose and glucose, had no inhibitory effect on the binding of the enzymes.

DISCUSSION. Unlike in earlier studies where only a single lysosomal enzyme has been used to demonstrate binding (13,14), in the present work we have shown that the brain receptor protein can bind at least four lysosomal enzymes: hexosaminidase, glucuronidase, fucosidase and arylsulfatase. The inability of mannosidase to bind is surprising and evidently points to the fact that either the requirement for the binding of this enzyme are not fully met by our isolated binding protein or that the mannosidase has a relatively lower affinity for binding and the other enzymes in the Concanavalin A-Sepharose fraction effectively compete with it precluding its binding. The latter possibility is more likely and will also account for the differing extent of binding by the lysosomal enzymes. The protein nature of the binding protein was borne out by the abolition of binding after pronase digestion.

Periodate treatment to a major extent and alkaline phosphatase to a limited extent abolishes the ability of the lysosomal enzymes to bind to the protein indicating the involvement of carbohydrates and more specifically a phosphorylated residue. However in addition to mannose-6-phosphate which was the most effective inhibitor, mannose and N-acetyl glucosamine were also capable of inhibition of the binding to a significant extent (Fig. 3). More than one possibility exists for this observation. a) In addition to mannose-6-phosphate, mannose and N-acetyl glucosamine moieties of the enzymes are also involved in the bind-

ing. b) Mannose and N-acetyl glucosamine resemble the actual recognition marker and therefore they can also reverse the binding. c) Mannose and /or N-acetyl glucosamine can also indepondently act as recognition markers. These observations are relevant to the recent findings of normal levels of lysosomal enzymes in the organs of patients with I-cell disease in spite of a deficient synthesis of the mannose-6-phosphate recognition marker (11, 12). It is also known that liver non parenchymal cells can internalize lysosomal enzymes via recognition of carbohydrate residues such as N-acetyl glucosamine or mannose (26). Moreover, it has been shown that in the high uptake form of glucuronidase from spleen, the majority of the mannose-6-phosphate residues were blocked by N-acetyl glucosamine (27), indicating the possible significance of this aminosugar in the uptake of lysosomal enzymes through receptors.

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