SPECIFICITY OF LOW MOLECULAR WEIGHT GLYCOPROTEIN EFFECTOR OF LIPID GLYCOSIDASE

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1. Introduction

The problem of control of activity of membrane-bound enzymes has not been adequately considered, in particular with regards to enzymes whose substrates are structural membrane components. Unregulated enzyme activity could have untoward consequences on membrane integrity and function. It has been proposed [1] that such enzymes are modulated by soluble effector proteins whose intracellular concentration and location are subject to cellular control; the special conditions under which the effector binds to the catalytic protein such as pH and availability of cofactors, could also be regulated in the best interest of the organism.

Such enzyme systems have been discovered [2–7] for a number of natural lipid hydrolases. One of these, the glucocerebroside β-glucosidase system, has been studied in some detail.

The system consists of a catalytic protein which requires for activity, a low mol. wt glycoprotein [1–4]. The glycoprotein effector is heat-stable and soluble; the catalytic protein is heat-labile and normally tightly membrane-bound [2,3]. Association between effector and catalytic proteins occurs at acid pH: incorporation of the effector into a membrane preparation of the catalytic protein is heat and time dependent, and parallels the appearance of enzyme activity in the membrane [2]. When the catalytic protein is solubilized [3,8], association with effector to produce active enzyme requires the addition of acidic membrane phospholipids, such as phosphatidic acid, phosphatidylserine and phosphatidylinositol [8]. In the presence of optimum concentrations of acidic phospholipid, the association reaches equilibrium instantaneously [1,9]. When the effector concentration is limiting, the association exhibits a 1:1 stoichiometry and proceeds essentially to completion. In the presence of excess of effector, a 2:1 complex is formed; this reaction does not go to completion and is characterised by a finite equilibrium constant [1,9].

So far, the available data indicate much similarity between effectors of different enzymes, in that they are heat-stable, low mol. wt acidic glycoproteins [2,5–7]. In order for such a control mechanism to be effective, some degree of specificity is required. In the ideal situation, one effector acts on one enzyme. This report describes the specific binding of the glucocerebrosidase catalytic protein to effector protein covalently linked to Sepharose.

2. Materials and methods

The effector protein was purified from the spleen of a patient with adult Gaucher's disease in which its concentration was increased 10-fold compared to controls. A 10% homogenate of spleen in distilled water was centrifuged (100 000 *g*, 1hr). The supernatant was heated at 100°C for 4 min, and the precipitated protein removed by centrifugation (1000 *g*, 15 min). The clear supernatant was concentrated 5-fold by ultrafiltration (Amicon ultrafiltration apparatus), then added to 9 vols of absolute ethanol at 0°C. The precipitate, collected by centrifugation (1000 *g*, 15 min) was redissolved in water and dialysed against 3 changes of 200 vols of distilled water overnight. The precipitate was removed by centrifugation (1000 *g*, 15 min). To the clear supernatant at 0°C was added dropwise

with mixing, 12% TCA to a final concentration of 2% (w/v). The precipitate was removed by centrifugation (1000 *g*, 15 min) and the supernatant dialysed overnight against 3 changes of 200 vols of water. The preparation was frozen for 48hr, then thawed, heated at 100°C for 4 min, and the precipitate removed by centrifugation. The supernatant was dialysed against 10 mM sodium phosphate buffer, pH 7.0, containing 40 mM NaCl. Gel filtration on Sephadex G-50 was carried out at room temperature in the same buffer. Fractions corresponding to apparent molecular weight of 10 000–20 000 were pooled and concentrated by ultrafiltration. The product ran as one diffuse PAS positive band on SDS gel electrophoresis [10] (Fig.1). The specific activity expressed as units of enzyme activity obtained in the presence of excess of catalytic protein was 101 000. One unit of enzyme activity represented the hydrolysis of 1 nmol substrate per hr at 37°C.

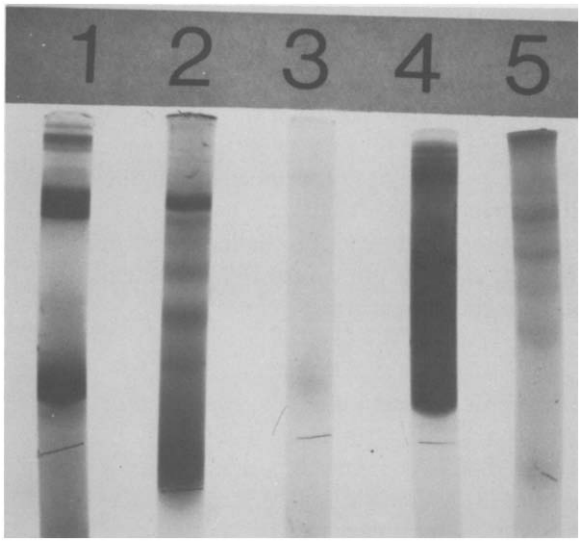

Fig.1. SDS gel electrophoresis. Samples (20–300 μg protein) were boiled for 5 min in 1% SDS + 1% mercaptoethanol then electrophoresed in 0.1% SDS-polyacrylamide gels [10]. Lane 1, Standards of bovine serum albumin (68 000) and cytochrome *c* (12 384) (both from Sigma, St. Louis Mo., USA); lane 2, heat inactivated 100 000 g supernatant from spleen of patient with Gaucher's disease; lane 3, purified effector protein; lane 4, ammonium sulphate precipitate of solubilized membrane protein from normal spleen (sample applied to effector-Sepharose column); lane 5, purified catalytic protein (pooled fractions 18–20, fig.2a). The position of the marker dye is indicated by insertion of a piece of steel wire before fixing and staining.

The purified effector was coupled to cyanogen-bromide activated agarose (CNBr-Sepharose 4B, Pharmacia, Uppsala, Sweden) using a procedure described by the manufacturer based on that of Cuatrecasas [11]. In two separate experiments, 5000 units and 10 000 units each of effector protein were reacted with 2 g of CNBr-Sepharose 4B. In both instances, over 95% of the protein was coupled. The effector-Sepharose (3.5 ml) was packed into a 5 × 1 cm column and washed with 4 column vols of buffer A (0.05 M sodium acetate, pH 4.5, containing 0.05% Triton X-100); then with 2 vols of buffer A containing 1 mg/ml phosphatidylserine.

A crude solubilized preparation of the catalytic protein from the total membrane fraction of normal human spleen was made as previously described [8]. This was then precipitated with 35 g/100 ml solid ammonium sulphate at 4°C. The precipitate obtained on centrifugation (10 000 *g*, 15 min) was dissolved in water and dialysed 8 hr against water and 8 hr against buffer A. The dialysed sample was mixed with phosphatidylserine (1 mg/10 ml) and applied to the column at room temperature. The flow rate was maintained at 15 ml per hr. The column was eluted sequentially with 4 or more column vols of the following buffers all containing 0.05% (v/v) Triton X-100: buffer A, buffer B (0.05 M sodium acetate, pH 5.3) and buffer C (0.02 M sodium phosphate, pH 7.0). Fractions of 3 ml were monitored for β-*N*-acetyl-glucosaminidase, β-galactosidase and glucocerebrosidase activities as described in detail elsewhere [3,8,12].

3. Results

A selective and quantitive binding of glucocerebrosidase catalytic protein was achieved. In column 1, the maximum activity bound was 4512 units (5000 units of effector coupled); in column 2, 9002 units (10 000 units coupled). This was a reasonable approximation to the 1:1 stoichiometry expected. The excess enzyme activity passed through unhindered. Fig.2a shows a profile of an experiment on column 2 with a non-saturating amount of catalytic protein. All the enzyme bound was eluted slowly with buffer C. A small amount of non-specifically bound β-*N*-acetyl-gluco-

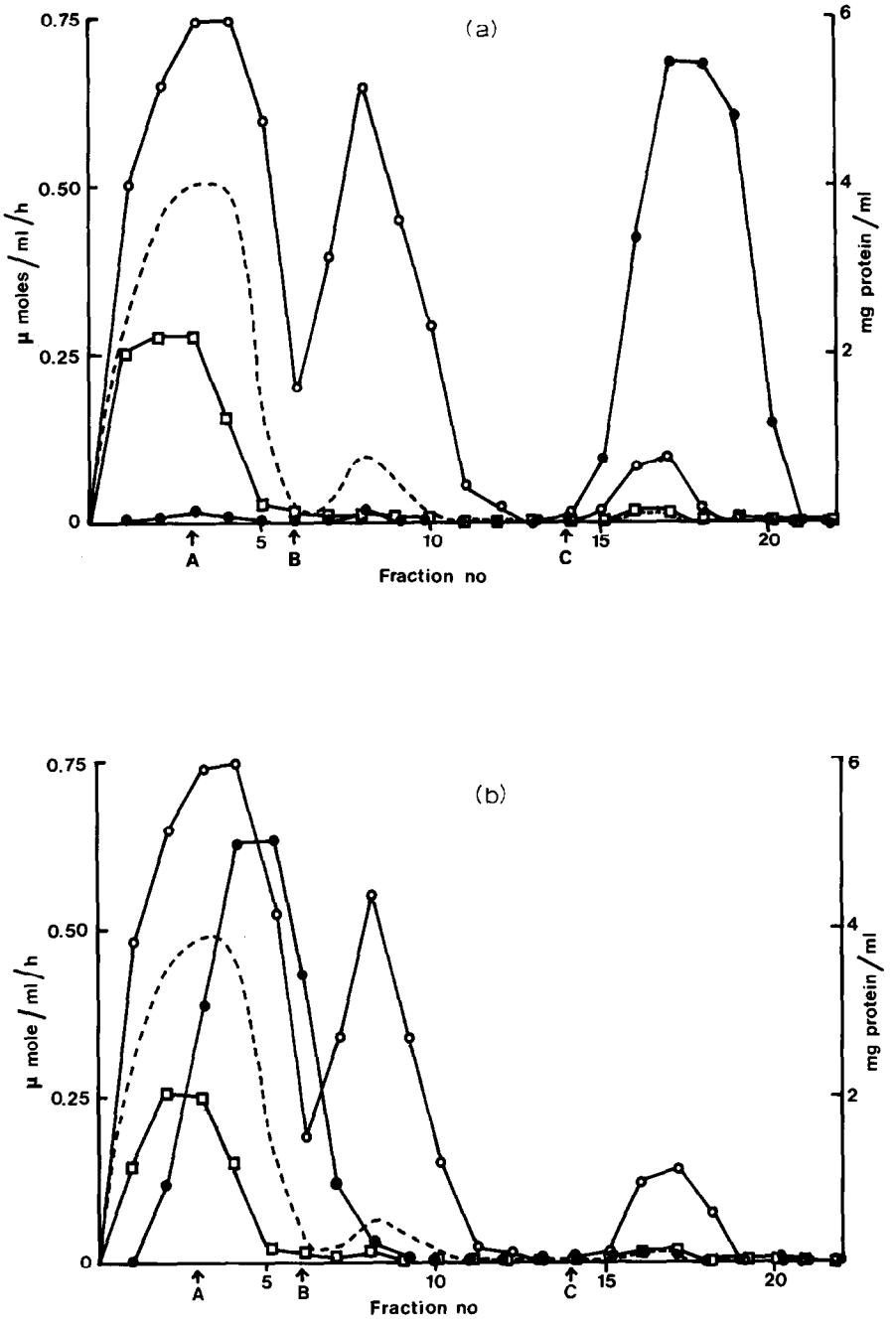

Fig.2. Chromatography of acid glycosidases. (a) Column of glucocerebrosidase effector coupled to Sepharose, (b) column of plain Sepharose. Conditions of elution are described in the text. β-*N*-Acetyl-glucosaminidase (○—○) (scaled down to one-tenth of real activity), and β-galactosidase (□—□) were assayed using the appropriate 4-methylumbelliferyl-glycoside derivatives [12] (Koch Light, Ltd., Poole, Dorset, UK). Glucocerebrosidase was assayed with the natural substrate, *N*-[^{14}C] stearoylglucosylsphingosine in the presence of excess of effector and 0.15 mM phosphatidylserine [8] (●—●). Arrows indicate switch overs in elution buffers A, B and C respectively (see text). Protein (– – –) was determined by the method of Lowry [13].

Table 1
Glycosidase activities in effector-Sepharose affinity chromatography

Glycosidase	Activity applied Total	Spec. act.**	Activity eluted* Total	Spec. act.**	Fold-purified
Glucocerebroside β-glucosidase	7584	158	4300	29 765	188.0
β-*N*-acetyl-glucosaminidase	179 520	3704	600	4152	1.1
β-Galactosidase	3456	72	3	21	0.3

* Pooled sample of fractions 18–20 from fig.2a
** Specific activity expressed as nmol/hr/mg protein
Conditions are as described in fig.2a.

saminidase partially overlapped with the peak of the glucocerebrosidase. Both β-*N*-acetyl-glucosaminidase and β-galactosidase activities had similar elution profiles on a column of plain Sepharose 4B from the same manufacturer (fig.2b). Glucocerebrosidase did not bind to plain Sepharose and was eluted in the first fractions with the buffer A wash (fig.2b). The purification of the catalytic protein, achieved by selective pooling to avoid contamination with β-*N*-acetyl-glucosaminidase, was 188-fold (table 1, fig.1).

Ammonium sulphate (10%) and low temperature (4°C) reduced binding 90%. Phosphatidic acid or phosphatidylinositol replaced phosphatidylserine completely to give a selective binding of glucocerebrosidase catalytic protein, in agreement with the previous finding [8] that all three phospholipids were effective in enabling association of the proteins in free solution. No binding of any of the three enzymes were obtained between pH 5.5–7.0, all activities co-eluted in the early fractions.

4. Discussion

These results demonstrate the specific binding of glucocerebrosidase catalytic protein to insolubilized effector protein under the same conditions that gave optimum association between the proteins in free solution [8]. The most likely explanation is that a specific effector exists for glucocerebrosidase. That this effector does not act on β-galactosidase, or β-*N*-acetyl-glucosaminidase is suggested by the lack of *selective* binding of these enzymes under the variety of conditions tested. The specificity for glucocerebrosidase is further attested to by the observation that the purified effector, far from stimulating hydrolysis of GM_1 ganglioside by β-galactosidase, strongly inhibited the reaction. Over 90% inhibition was achieved with 1 mg/10 ml effector. The application of effector-Sepharose in enzyme purification is being explored.

Acknowledgement

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