Human placental β -galactosidase

Characterization of the dimer and complex forms of the enzyme

Martin HUBBES,*† Raffaele M. D'AGROSA*† and John W. CALLAHAN*†‡§

* Research Institute, The Hospital For Sick Children, and Departments of † Biochemistry and ‡ Pediatrics, University of Toronto, Toronto, Ontario M5G 1X8, Canada

G_{M1} ganglioside β -galactosidase (β -Gal) is deficient in the autosomal recessive disorder G_{M1} gangliosidosis. A portion of the enzyme occurs in a complex with neuraminidase and an additional glycoprotein, protective protein, but the nature of the interactions conferring the stability of the complex is unknown. Affinity chromatography of β -Gal on *p*-aminophenylthiogalactose–Sepharose (PATG–Sepharose) at pH 4.3, the pH optimum of β -Gal, resulted in a 260-fold enrichment of β -Gal, but the major protein in the fraction had an M_r value of 74000. Affinity chromatography on PATG–Sepharose at pH 5.2 showed substantial enrichment (4000-fold) of β -Gal, and the mature form of the enzyme (M_r 64000) was the major protein in the preparation. Using h.p.l.c. molecular-sieve chromatography, we found that about 15% of the total β -Gal occurred in a high- M_r form ($> 600\,000$), the presumptive complex, with 85% eluting at M_r 150000, suggestive of a dimer. This distribution was independent of both high (60 mg/ml) and low (5 mg/ml) protein concentration and the pH (pH 4.3 or 5.2) of the sample applied to the column. Furthermore, incubation for 90 min at 37 °C, conditions which had previously been suggested as optimal for formation of the complex, had no effect on this distribution. Further fractionation by anion-exchange chromatography and a second affinity column step yielded a β -Gal preparation that contained a single polypeptide chain (M_r 64000), was devoid of neuraminidase and protective protein (absent carboxypeptidase activity), and when injected into rabbits gave rise to monospecific rabbit antisera. We conclude that the protein composition of the complex is variable (i.e. it is different when isolated at pH 4.3 and 5.2) and that the amount of β -Gal tightly associated with the complex constitutes a small fraction of the total β -Gal activity. The more prevalent form of the enzyme is a β -Gal homodimer that is stable and devoid of either neuraminidase activity or protective protein.

INTRODUCTION

G_{M1} ganglioside β -galactosidase (EC 3.2.1.23; β -Gal) is a lysosomal enzyme that hydrolyses the β 1-4 or β 1-3 linkage between terminal galactose residues of oligosaccharides (Alpers, 1969; Asp, 1971) and glycolipids (Gatt, 1967). The main substrate in brain is G_{M1} ganglioside (Okada & O'Brien, 1968). The purification of lysosomal β -Gal was first reported from human liver (Norden & O'Brien, 1974). Multiple forms of liver β -Gal were resolved on molecular-sieve columns, one corresponding to a high M_r (about 800000) and the other corresponding to a 65000- M_r monomer. It was initially proposed that the 800000- M_r form, and an intermediate form (M_r 150000) seen in liver, were simply aggregates of the 65000- M_r monomer (Cheetham & Dance, 1976). However, Frost *et al.* (1978) showed that the 800000- M_r form was composed of four major proteins, with M_r values of 65000, 32000, 22000 and 21000. In 1982, Verheijen *et al.* (1982) demonstrated the presence of neuraminidase activity in this fraction and proposed the existence of a complex. To date, three proteins have been identified as components of the complex, namely β -Gal (monomer M_r 64000), neuraminidase (monomer M_r 66000) and a third protein, called protective protein (native monomer M_r 54000; on reduction this resolves into 32000- and 20000- M_r components) which 'protects' β -Gal and neuraminidase from proteolytic digestion (Van Diggelen *et al.*, 1981, 1982; D'Azzo *et al.*, 1982; Verheijen *et al.*, 1985; Takeda *et al.*, 1986; Nanba *et al.*, 1987). It was recently shown that

protective protein possesses lysosomal serine protease activity similar to that of the well characterized carboxypeptidase Y of yeast (D'Azzo, 1990).

We (D'Agrosa & Callahan, 1988) have shown that neuraminidase activity of the complex can be significantly enhanced by digestion with exogenously added cathepsin C, while under the same conditions exogenous cathepsin D completely destroyed the enzyme. β -Gal was completely unaffected by both enzymes under the same conditions. However, after further enrichment of β -Gal which resulted in removal of neuraminidase activity and protective protein, β -Gal became susceptible to the action of these cathepsins (D'Agrosa & Callahan, 1988). This raises the interesting questions of what types of interactions involving β -Gal stabilize the complex, and what proteins are required for this stability. To approach the answers to some of these questions, we sought separately to purify the complex containing β -Gal, and β -Gal alone. We found that the complex contains only 15–20% of the total β -Gal activity, while the bulk of the enzyme occurs as a dimeric enzyme. A preliminary report of this work has appeared (Callahan *et al.*, 1990).

MATERIALS AND METHODS

Analytical procedures

β -Gal activity was measured by using 4-methylumbelliferyl β -D-galactopyranoside (4MU-gal) (Koch–Light laboratories)

Abbreviations used: β -Gal, β -galactosidase; PATG, *p*-aminophenylthiogalactose; 4MU-gal, 4-methylumbelliferyl β -D-galactopyranoside; 4-MU-NeuAc, 4-methylumbelliferyl *N*-acetylneuraminic acid; DTT, dithiothreitol; ID, internal diameter; TBS, tris-buffered saline; HRP, horseradish peroxidase; Con A, concanavalin A.

§ To whom correspondence should be addressed, at: Division of Neurosciences, Research Institute, The Hospital for Sick Children, 555 University Avenue, Toronto, Ontario, M5G 1X8, Canada.

(Jones *et al.*, 1984). Briefly, 190 μ l of substrate solution (0.1 M-citrate buffer, pH 4.3; 0.56 mM-4MU-gal) were mixed with 60 μ l of enzyme/water and incubated for 30 min at 37 °C. The reaction was terminated with the addition of 2 ml of 0.1 M-2-amino-2-methyl-1-propanol buffer, pH 10 (MAP) and the fluorescence was determined with an excitation wavelength of 365 nm and an emission wavelength of 450 nm on either a Turner (model 430) or a Perkin-Elmer (model 650-40) spectrofluorimeter. Assays were carried out in duplicate. 4-Methylumbelliferone (Eastman) solution was used as a standard. Neuraminidase activity was estimated by using the artificial substrate 4-methylumbelliferyl *N*-acetylneuraminatate (4MU-NeuAc) (O'Brien & Warner, 1980). The protein content of solutions was estimated by the Folin-phenol method (Lowry *et al.*, 1951) using crystalline BSA as a standard.

Enrichment of β -Gal

Twenty human placentae were washed under running tap water, stripped of outer membranes, cut into strips and passed through a meat grinder (Braun model KGZ3). All subsequent procedures were carried out in a cold room at 4 °C. The ground placentae were homogenized in 10 l of 10 mM-phosphate buffer, pH 6.0, with 10^{-4} M-dithiothreitol (DTT) in a 4 l blender (Waring model 91-215). The resulting homogenate was then stored overnight (12 h), after which time it was centrifuged at 3000 *g* for 1 h.

Concanavalin A (Con A) and Sephadex G-25 column chromatography. The supernatant (approx. 10 l) was loaded on to a 500 ml Con A-Sepharose (Pharmacia) column (height 18 cm; diameter 6 cm; capacity 20 mg protein/ml) equilibrated with 10 mM-phosphate buffer, pH 7.0, 10^{-4} M-DTT and 0.5 M-NaCl (buffer A). The column was washed with 2 l of buffer A and then eluted with 1.5 l of buffer A containing 15% (w/v) methyl α -D-mannopyranoside (Sigma). The eluant was recycled through the column by a peristaltic pump (Pharmacia model p1) for 3 days. The column was finally washed with a further 0.5 l of buffer A containing 10% (w/v) methyl α -D-mannopyranoside, which was pooled with the first 1.5 l of eluate. The Con A eluate was concentrated to 1 l by using a hollow-fibre concentrator (Amicon model LP-1) and was then loaded on to a 10 l Sephadex G-25 (Pharmacia, coarse grade) column pre-equilibrated with 1% (w/v) saline in order to separate the protein from the methyl α -D-mannopyranoside. The void volume of the column (2200 ml) was discarded and the enzyme was collected in the next 1800 ml of eluate.

Hexosaminidase affinity chromatography. The G-25 column eluate was concentrated to 600 ml, and split into three 200 ml portions. A 200 ml aliquot was loaded on to the 800 ml hexosaminidase affinity column [2-acetamido-*N*-(ϵ -aminocaproyl)-2-deoxy- β -D-glucopyranosylamine coupled to Sepharose S-200] (Mahuran & Lowden, 1980) pre-equilibrated with 10 mM-phosphate buffer, pH 6.0, 10^{-4} M-DTT and 0.5 M-NaCl (buffer B). The unbound fraction containing the bulk of the β -Gal activity was collected. The column was stripped of bound hexosaminidase with 1 l of 10 mM-Tris/HCl buffer, pH 8.0, and 0.5 M-NaCl, and was re-equilibrated with 2 l of buffer B. The procedure was repeated for the other two aliquots.

Isolation of homogeneous β -Gal

Affinity chromatography. The unbound fraction from the hexosaminidase affinity column (0.5 l) was acidified to pH 5.2 by the dropwise addition of 1.0 M-acetic acid and was clarified by centrifugation at 3000 *g* for 30 min. The supernatant was applied to a 20 ml PATG-Sepharose β -Gal affinity column which had

been pre-equilibrated with 20 mM-acetate buffer, pH 5.2, 10^{-4} M-DTT and 300 mM-NaCl (buffer D). The PATG-Sepharose column was prepared according to the manufacturer's (Pharmacia) instructions. Briefly, 15 g of dried powder CH-Sepharose gel was swollen in water to a volume of about 45 ml, 300 mg of *p*-aminophenylthiogalactoside ligand was added, followed by *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride to a concentration of 0.1 M, the pH was adjusted to 5.0, and the final volume was 100 ml. The column was washed with 200 ml of buffer D and the enzyme was eluted with 50 ml of 10 mM-Tris/HCl buffer, pH 7.0, 10^{-4} M-DTT, 1.0 M-NaCl and 0.1 M- γ -D-galactonolactone (Sigma).

The eluate was dialysed against 4 l of 10 mM-phosphate buffer, pH 6.0, and 10^{-4} M-DTT (buffer E) for 12 h. Proteins were loaded on to a 20 ml DEAE-Sephadex A 50 column, washed thoroughly with a further 200 ml of buffer E, and then eluted with a linear 0–0.4 M-NaCl gradient in the same buffer. The eluate was collected in 4.5 ml fractions and was analysed for enzyme activity.

Fractions containing β -Gal activity were pooled, concentrated to 5 ml, and dialysed against 2 l of buffer D. This concentrate was then applied to a second, smaller (2 ml) PATG affinity column which had been pre-equilibrated with buffer D. This column was eluted as described above.

Isolation of different forms of the complex by affinity chromatography

Affinity chromatography of the enzyme has included the use of citrate buffer, at pH 4.3, and acetate buffer at pH 5.2 (Lo *et al.*, 1979; Verheijen *et al.*, 1982; Jones *et al.*, 1984). To determine if the composition of the β -Gal pool was the same under these different affinity column conditions, the unbound fraction from the hexosaminidase affinity column (designated fraction Gp) was concentrated to a volume of 0.5 l by using the hollow-fibre concentrator. Approximately one-half of fraction Gp was acidified to pH 4.3 by the dropwise addition of 1.0 M-citric acid, and an inactive pellet was removed by centrifugation at 3000 *g* for 30 min. The supernatant (called Gp4.3) was loaded on to a 20 ml affinity column pre-equilibrated with 0.1 M-citrate buffer, pH 4.3, 10^{-4} M-DTT and 0.5 M-NaCl (buffer C). The column was washed with 250 ml of buffer C and the enzyme solution (called p4.3) was eluted with 50 ml of buffer C containing 0.5 M- γ -D-galactonolactone.

The other portion of Gp was acidified to pH 5.2 by the dropwise addition of 1.0 M-acetic acid and an inactive pellet was removed as above. The supernatant (designated Gp5.2) was applied to a separate 20 ml PATG affinity column which had been previously equilibrated with 20 mM-acetate buffer, pH 5.2, 10^{-4} M-DTT and 300 mM-NaCl (buffer D). The column was washed with 200 ml of buffer D and the enzyme (called p5.2) was eluted with 50 ml of 10 mM-Tris/HCl buffer, pH 7.0, 10^{-4} M-DTT, 1.0 M-NaCl and 100 mM- γ -D-galactonolactone.

H.p.l.c. gel filtration of β -Gal

Sample preparation. In this experiment one-half of Gp was concentrated to 60 mg/ml by using the hollow-fibre concentrator and an Amicon pressure concentrator (50 ml) fitted with a YM10 membrane, while the other half was diluted to a protein concentration of 5 mg/ml. One-half of the 60 mg/ml and 5 mg/ml samples was separately acidified to pH 5.2 (Gp5.2) by the dropwise addition of 1.0 M-acetic acid, and the other half of the 60 mg/ml and 5 mg/ml samples was brought to pH 4.3 (Gp4.3) by the addition of 1.0 M-citric acid. The solutions were then cleared of any precipitate by centrifugation at 3000 *g* for 30 min. Each of the resulting four solutions was then split again into two

equal parts, half being stored at 4 °C while the other half was incubated at 37 °C for 90 min.

H.p.l.c. gel filtration and analysis. The samples were chromatographed at room temperature on three TSK 3000 molecular-sieve columns in series (Altex Spherogel 7.5 mm internal diam. $\times$ 30 cm) equilibrated with 0.1 M-phosphate buffer, pH 6.0, and 0.5 M-NaCl. All of the 60 mg/ml samples were injected using a 20 μ l sample loop while all the 5 mg/ml samples were loaded with a 250 μ l loop. The columns were eluted at a flow rate of 0.5 ml/min and each fraction represents a 1-min collection. Aliquots (16 μ l) from each fraction were assayed for β -Gal activity as described. Similarly, fractions p4.3 and p5.2 from the affinity columns were chromatographed. The results were plotted and the area under each activity peak was calculated using the Curves program for the Apple MacIntosh computer.

Antisera against β -Gal

Antisera were raised in two New Zealand rabbits. Each was immunized with an initial intramuscular injection with 150 μ g of homogeneous β -Gal coupled to KAl(SO₄)₂ (Williams & Chase, 1967), followed every 2 weeks by injection of 30–50 μ g of homogeneous β -Gal suspended in 100 μ l of Freund's incomplete adjuvant. Blood was allowed to clot at 4 °C and sera were collected and stored at –70 °C until used.

PAGE and Western blots

SDS/PAGE was carried out according to the method of Laemmli (1970), using a 16 cm $\times$ 18 cm $\times$ 1.5 mm slab gel apparatus (Bio-Rad) or a 8 cm $\times$ 10 cm $\times$ 0.3 mm minigel apparatus (Bio-Rad). Samples were denatured in sample buffer [0.625 M-Tris/HCl, pH 6.8, 20% glycerol, 50 mM-DTT, 3% (w/v) SDS and 0.001% Bromophenol Blue] either by heating them to 100 °C for 10 min or by incubating them at 60 °C for 20 min. M_r was determined by using the Pharmacia M_r standards. Gels were stained either with silver stain (BioRad kit) or with Coomassie Brilliant Blue R250 (Sigma).

Proteins were transferred from the unfixed gel on to the nitrocellulose (Towbin *et al.*, 1979). The unbound sites on the nitrocellulose were blocked with 0.25% gelatin in Tris-buffered saline (TBS; 0.15 M-NaCl, 50 mM-Tris buffer, pH 7.4) for 4 h and then incubated overnight at room temperature with 1:500 diluted antiserum in a solution of blot buffer (TBS containing 0.25% gelatin, 0.05% Nonidet P-40 and 5 mM-EDTA). The nitrocellulose was washed for 4 h in blot buffer (three changes) and then incubated overnight in horseradish peroxidase (HRP)-labelled goat anti-rabbit IgG diluted 1:1000 in blot buffer. After having been washed for 4 h, the immunoreactive proteins were visualized by the addition of stain solution, which consists of two freshly mixed solutions (60 mg of 4-chloro-1-naphthol in 20 ml of ice-cold methanol, and 60 μ l of 30% H₂O₂ in 100 ml of TBS). Colour development was stopped by washing with distilled water.

RESULTS

β -Gal-neuraminidase-protective protein complex

Affinity chromatography of β -Gal has employed buffers principally in the pH range 4.3–5.2 but no one to date has directly compared the resulting preparations. To do this, a preparation of β -Gal was brought to the affinity column stage, split into two equal pools, and acidified to pH 4.3 to give Gp4.3 and to pH 5.2 to give Gp5.2 as described in the Materials and methods section. The protein composition of the samples and specific activities of various lysosomal hydrolases in these fractions prior to affinity column chromatography were comparable (Table 1), while the

enzyme and the protein composition of the samples p4.3 and p5.2 eluted from the affinity column were different (Fig. 1). The p5.2 eluate showed an abundance of β -Gal (M_r 64000), and lower amounts of the 32000- and 20000- M_r protective protein polypeptides indicative of the presence of the complex (Fig. 1a). The p4.3 eluate showed decreased amounts of β -Gal and the other components of the complex (Fig. 1b), with the most abundant protein having M_r 74000. Two attempts to determine an N-terminal sequence of the 74000- M_r protein were unsuccessful, in spite of adequate amounts of protein, as judged by amino acid analysis. Others (Verheijen *et al.*, 1987) have found the same protein but its identity is currently unknown.

Elution of the affinity column at pH 5.2 resulted in a 20-fold higher enrichment of β -Gal than that found in the corresponding eluate at pH 4.3. Neuraminidase activity was about 6-fold higher in the p5.2 eluate but other hydrolases, such as α -mannosidase, and β -hexosaminidase were much higher in p4.3 than in p5.2 (Table 1). Interestingly, galactocerebrosidase, a genetically and structurally distinct form of β -Gal, was about 290-fold higher in specific activity in the p4.3 eluate than in the p5.2 preparation. Its

Table 1. Comparison of specific activities of lysosomal hydrolases

The specific activity of several lysosomal hydrolases was measured in the glycoprotein fractions applied to the affinity column (Gp5.2 and Gp4.3) and in the fractions eluted from it (p5.2 and p4.3). ND indicates that the activity level found was too low to be detected.

Enzyme	Fraction ...	Specific activity (μ mol $\cdot$ h ⁻¹ $\cdot$ mg of protein ⁻¹)			
		Gp5.2	Gp4.3	p5.2	p4.3
GM ₁ β -Gal		1.5	2.0	203	11
Neuraminidase		0.003	0.01	0.3	0.05
Galactocerebrosidase		0.2	0.2	0.1	29
β -Mannosidase		0.6	1.2	0.5	0.6
α -Mannosidase		0.03	0.8	ND	8.3
β -Hexosaminidase		670	740	42	2100

Fig. 1. SDS/PAGE of (a) the p5.2 eluate and (b) the p4.3 eluate from the PATG column

(a) Lane ST was loaded with 20 μ g of Pharmacia M_r markers. Lanes 1 and 2 were loaded with 59 μ g of protein eluted from the column. The major peptide observed had M_r 64000. (b) Lanes ST were loaded with 20 μ g of Pharmacia M_r standards. Lanes 1, 2 and 3 were loaded with 30, 20 and 10 μ g respectively of the eluted fraction.

Fig. 2. Elution profile of Gal activity on h.p.l.c. columns

Glycoprotein fractions were loaded on to three TSK 3000 molecular-sieve h.p.l.c. columns. Fractions Gp4.3 and Gp5.2 were prepared as described.

Table 2. Elution of β -Gal activity on h.p.l.c.

Fractionation was carried out as described in the legend to Fig. 2. The amount of β -Gal activity was calculated using the MacIntosh Curves program.

Sample	Peak no.	Area (% of total)
60 mg/ml, pH 5.2, 37 °C	1	18
	2	81
60 mg/ml, pH 5.2, 4 °C	1	10
	2	89
5 mg/ml, pH 5.2, 37 °C	1	11
	2	88
5 mg/ml, pH 5.2, 4 °C	1	14
	2	85
60 mg/ml, pH 4.3, 37 °C	1	17
	2	81
60 mg/ml, pH 4.3, 4 °C	1	14
	2	85
5 mg/ml, pH 4.3, 37 °C	1	21
	2	78
5 mg/ml, pH 4.3, 4 °C	1	18
	2	81

polypeptide has an M_r of about 120000 (M. Hubbes & J. W. Callahan, unpublished work).

Since the distribution of the enzymes in the affinity column eluates varied, we wondered if the relative amount of the complex prior to affinity chromatography was also subject to variation. It has been reported that high protein concentrations (60 mg/ml) and heating to 37 °C for 90 min were required to obtain optimal amounts of β -Gal in the β -Gal-neuraminidase-protective protein complex. We assessed this by subjecting glycoprotein fractions to different conditions including acidification pH (either pH 4.3 or 5.2), protein concentration (5 and 60 mg/ml), or incubation temperature (37 or 4 °C) and then fractionating the enzyme on TSK 3000 h.p.l.c. molecular-sieve columns. The β -Gal activity always eluted in two peaks (Fig. 2). The first peak (peak 1) was eluted in the void volume ($M_r > 800000$), while the second (peak

Fig. 3. (a) SDS/PAGE of purified β -Gal; (b) Western blot of β -Gal and (c) Western blot of glycopeptidase F-digested β -Gal

(a) Purified β -Gal eluted from the small 3 ml PATG column was analysed by SDS/PAGE on a 12% gel. Lane ST was loaded with 10 μ g of Pharmacia M_r standards. Lane 1 was loaded with 5 μ g of purified β -Gal. The gel was silver stained to visualize the proteins. (b) The p5.2 affinity column eluate was run on a 12% SDS/PAGE gel and blotted on to nitrocellulose. IgG, recovered from a preparative nitrocellulose slice corresponding to the zone of β -Gal (lane 1), was incubated with the p5.2 affinity column eluate (lane 2). There is a clear reaction against the 64000- M_r polypeptide and two lower- M_r bands presumed to be degradation products of the mature protein. On additional testing they showed approximate M_r values of 43000 and 29000. (c) Purified β -Gal was digested with glycopeptidase F (Mahuran *et al.*, 1988), run on an SDS/12%-PAGE gel, and transblotted on to nitrocellulose. The blots were incubated in antibody and visualized using HRP linked to goat anti-rabbit IgG. Lane 1 was loaded with undigested β -Gal and lane 2 was loaded with the digested material. The apparent M_r of the deglycosylated peptide is 59000.

2) eluted at 65 min corresponding to M_r 152000. Under all conditions examined (Table 2) the amount of β -Gal present in the high- M_r complex was always approx. 15% (range of values 10–21%) of the total activity, while the other 85% eluted with a mobility consistent with a dimeric form of the enzyme.

Isolation of homogeneous β -Gal

To determine if neuraminidase or protective protein contaminate the 150000- M_r form of the enzyme, the p5.2 affinity column eluate was chromatographed on an anion-exchange column. Two peaks of β -Gal were resolved: one, eluting at low NaCl concentrations and comprising about 20% of the total enzyme activity, contained both β -Gal and the other proteins of the complex, while the bulk of the activity eluted in a very broad band at NaCl concentrations of 0.17–0.25 M (results not shown). This eluate was concentrated by passage through a second, small affinity column. The homogeneous enzyme had an M_r of 134000 on h.p.l.c., and gave a single polypeptide of M_r 64000 on SDS/PAGE gels (Fig. 3a). This preparation showed a specific activity of 660 μ mol $\cdot$ h $^{-1}$ $\cdot$ mg $^{-1}$, and was devoid of neuraminidase, carboxypeptidase Y activity and hexosaminidase activity.

Antisera raised in rabbits against this preparation precipitated β -Gal activity and both the 64000- M_r polypeptide and the glycopeptidase F-digested polypeptide (M_r 59000) reacted strongly with the rabbit antibody (Figs. 3b and 3c). Minor amounts of two peptides, one near M_r 43000–45000 and the second near M_r 29000, were sometimes visualized (results not shown). These are likely to be breakdown products of β -Gal since the enzyme was always derived from pooled affinity column eluates and, in one instance where these two peptides were prominent, represented enzyme from 120 placentae (Fig. 3).

DISCUSSION

The complex high- M_r form of β -Gal constituted 15% of the total β -Gal activity, while about 85% of the enzyme occurred as a dimer with M_r near 134000. These results are in agreement with very early reports suggesting that about 20% of total β -Gal is found as a high- M_r form (Norden *et al.*, 1974; Cheetham & Dance, 1976). We obtained no evidence that acidifying the enzyme solution, concentrating it to 60 mg/ml, or incubating it at 37 °C for 90 min increased measurably the amount of complex. We have previously shown that this treatment dramatically increased the amount of neuraminidase activity in the sample but we found that this was due to a proteolytic activation of the neuraminidase during the concentration and incubation (D'Agrosa & Callahan, 1988) rather than to an increase in the amount of complex. Previous data have suggested the neuraminidase has a significant content of complex carbohydrate and it is likely, although not proven, that association of dimeric β -Gal with the complex involves conformational changes which mask the enzymes from the action of proteases. That protective protein has carboxypeptidase Y activity was predicted by Galjart *et al.* (1988, 1990) based on the similarity of the deduced sequence of protective protein with those of the yeast carboxypeptidase Y and KEX 1 enzymes. We have found carboxypeptidase activity in the high- M_r complex fraction and have direct evidence that the protective protein functions as a carboxypeptidase (D'Agrosa *et al.*, 1992).

To rule out the presence of neuraminidase and the protective protein in the dimeric 134000- M_r form of β -Gal, we subjected the enzyme to further fractionation. The final specific activity of the enzyme was high when using both artificial and natural substrates, the preparation was devoid of carboxypeptidase Y and neuraminidase activity, and there was no evidence of these proteins on SDS/PAGE gels. The purified preparation showed a single polypeptide of M_r 64000, consistent with the size of β -Gal deduced from cDNA (Oshima *et al.*, 1988). Rabbit antisera raised against the protein were highly selective for native β -Gal, the 64000- M_r monomer, and the deglycosylated 59000- M_r polypeptide. The precursor of the enzyme was never more than a minor component in any of our preparations. Small amounts of immunoreactive protein components with M_r values of 43000 and 29000 were detected on Western blots, initially when affinity-purified IgG was used, and larger amounts of material were seen in the M_r 43000 to 45000 range in a pool of homogeneous enzyme obtained over the course of several months from about 120 placentae. These are presumed to be breakdown products of the enzyme. We are unaware of any reports of active 43000- M_r forms of the enzyme, but Kress & Miller (1978) did report the isolation of an active form of β -Gal that had M_r 27000 from human urine. As a further proof of the specificity of these antisera, tissues and fibroblasts from patients with type I G_{M1} -gangliosidosis have been shown to have markedly reduced amounts of cross-reacting material (Callahan *et al.*, 1990; D'Agrosa *et al.*, 1992).

Our data indicating that the homodimer of β -Gal is the most abundant form of the enzyme are in complete accord with the requirements for trafficking of the enzyme in the cell, a subject reviewed by Lodish (1988). On this basis, the polypeptide-polypeptide interactions that give rise to dimeric β -Gal must occur prior to and as a requirement for its exit from the endoplasmic reticulum. Since, after removal of the signal peptide, little if any post-translational processing of the enzyme would

occur prior to its localization in the lysosome, dimeric unprocessed β -Gal would be expected to be the major form of the enzyme prior to its interaction with the protective protein and neuraminidase to form the complex. Our data are in complete accord with this expectation.

This work was supported by a grant from the Medical Research Council of Canada. We are indebted to Dr. Don Mahuran for assistance in the preparation of this manuscript and to Amy Leung for help in the early steps of the purification procedure.

REFERENCES

- Alpers, D. H. (1969) *J. Biol. Chem.* **244**, 1238–1246
 Asp, N. G. (1971) *Biochem. J.* **121**, 299–308
 Callahan, J. W., D'Agrosa, R. M., Hubbes, M., Shankaran, R. & Novak, A. (1990) *Trans. Am. Soc. Neurochem.* **21**, 127
 Cheetham, P. S. J. & Dance, N. E. (1976) *Biochem. J.* **157**, 189–195
 D'Agrosa, R. & Callahan, J. W. (1988) *Biochem. Biophys. Res. Commun.* **157**, 770–775
 D'Agrosa, R. M., Hubbes, M., Zhang, S.-Q., Shankaran, R. & Callahan, J. W. (1992) *Biochem. J.* **285**, 833–838
 D'Azzo, A. (1990) *Int. Congr. Inborn Errors Metab.* **5**, II.6
 D'Azzo, A., Hoogeveen, A., Reuser, A. J. J., Robinson, D. & Galjaard, H. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 4535–4539
 Frost, R. J., Holmes, E. W., Norden, A. G. W. & O'Brien, J. S. (1978) *Biochem. J.* **175**, 181–188
 Galjart, N. J., Gillemans, N., Harris, A., Van Der Horst, G. T. J., Verheijen, F. W., Galjaard, H. & D'Azzo, A. (1988) *Cell* **54**, 755–764
 Galjart, N. J., Gillemans, N., Meijer, D. & D'Azzo, A. A. (1990) *J. Biol. Chem.* **265**, 4678–4684
 Gatt, S. (1967) *Biochim. Biophys. Acta* **137**, 192–195
 Jones, C. S., Mahuran, D., Lowden, J. A. & Callahan, J. W. (1984) *Can. J. Biochem.* **62**, 529–534
 Kress, B. C. & Miller, A. L. (1978) *Clin. Chim. Acta* **85**, 23–32
 Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
 Lo, J. T., Mukerji, K., Awasthi, Y. C., Hanada, E., Suzuki, K. & Srivastava, S. K. (1979) *J. Biol. Chem.* **254**, 6710–6715
 Lodish, H. F. (1988) *J. Biol. Chem.* **263**, 2107–2110
 Lowry, O. H., Rosebrough, J. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
 Mahuran, D. & Lowden, J. A. (1980) *Can. J. Biochem.* **58**, 287–294
 Mahuran, D. J., Neote, K., Klavins, M. H., Leung, A. & Gravel, R. A. (1988) *J. Biol. Chem.* **263**, 4612–4618
 Nanba, E., Tsuji, A., Omura, K. & Suzuki, Y. (1987) *J. Clin. Biochem. Nutr.* **3**, 149–157
 Norden, A. G. W. & O'Brien, J. S. (1974) *Biochem. Biophys. Res. Commun.* **56**, 1973–1993
 Norden, A. G. W., Tennant, L. L. & O'Brien, J. S. (1974) *J. Biol. Chem.* **249**, 7969–7976
 O'Brien, J. S. & Warner, T. (1980) *Clin. Genet.* **17**, 35–38
 Okada, S. & O'Brien, J. S. (1968) *Science* **160**, 1002–1004
 Oshima, A., Tsuji, A., Nagao, Y., Sakuraba, H. & Suzuki, Y. (1988) *Biochem. Biophys. Res. Commun.* **157**, 238–244
 Takeda, E., Kuroda, Y., Toshima, K., Naito, E., Ito, M., Miyao, M., Kominami, E. & Katunuma, N. (1986) *Clin. Chim. Acta* **155**, 109–116
 Towbin, H., Staehlin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4355
 Van Diggelen, O. P., Schram, A. W., Sinnott, M. L., Smith, P. J., Robinson, D. & Galjaard, H. (1981) *Biochem. J.* **200**, 143–151
 Van Diggelen, O. P., Hoogeveen, A. T., Smith, P. J., Reuser, A. J. & Galjaard, H. (1982) *Biochim. Biophys. Acta* **703**, 69–76
 Verheijen, F. W., Brossmer, R. & Galjaard, H. (1982) *Biochem. Biophys. Res. Commun.* **108**, 868–875
 Verheijen, F. W., Palmeri, S., Hoogeveen, A. T. & Galjaard, H. (1985) *Eur. J. Biochem.* **149**, 315–321
 Verheijen, F. W., Palmeri, S. & Galjaard, H. (1987) *Eur. J. Biochem.* **162**, 63–67
 Williams, C. A. & Chase, M. W. (1967) *Methods in Immunology and Immunochemistry*, Academic Press, New York