α -Fucosidase–ganglioside interactions.

Action of α -L-fucosidase from the hepatopancreas of *Octopus vulgaris* on a fucose-containing ganglioside (Fuc-GM1)

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α -L-Fucosidase, prepared in highly purified form (M_r 70000–74000) from *Octopus* hepatopancreas, was able to hydrolyse a fucose-containing ganglioside, namely Fuc-GM1 (II³NeuAc,IV²Fuc-GgOse₄-Cer). The enzyme showed an irregular kinetic behaviour ($v/[S]$ and $v/[E]$ relationships following sigmoidal curves) when working on micellar Fuc-GM1 (M_r of the micelle 500000), but obeyed regular hyperbolic kinetics when acting on low- M_r substances. It was observed that, on incubation with micellar Fuc-GM1 under the conditions used for the enzyme assay, *Octopus* α -L-fucosidase produced a ganglioside–enzyme complex that was catalytically inactive. This complex had an M_r exceeding 500000 and a ganglioside/protein ratio of 4:1 (w/w), which is consistent with a stoichiometric combination of one ganglioside micelle with two enzyme molecules. Inactivation of α -L-fucosidase by formation of the corresponding complexes was also obtained with micellar gangliosides GM1 (II³NeuAc-GgOse₄-Cer), GD1a (II³NeuAc,IV³NeuAc-GgOse₄-Cer) and GT1b [II³(NeuAc)₂,IV³-NeuAc-GgOse₄-Cer], which are not substrates for the enzyme, indicating that the ganglioside micelles *per se* act as enzyme inhibitors. However, α -L-fucosidase easily forms a Fuc-GM1– α -L-fucosidase complex, displaying regular Michaelis–Menten kinetics. Therefore the anomalous behaviour exhibited by α -L-fucosidase on micellar Fuc-GM1 is likely due to formation of the complex, which separates the fucosyl linkage from the active site of the complexed enzyme, but makes it available to the enzyme in the free form.

α -L-Fucosidase occurs in bacteria, protozoa, moulds and marine organisms and seems to be ubiquitous in mammalian fluids and tissues (Alhadeff & O'Brien, 1977). The enzyme removes fucose α -glycosidically linked to oligosaccharides, glyco-

lipids, glycoproteins or to synthetic aglycones (Kochibe, 1973; Reglero & Cabezas, 1976).

The relationships between α -L-fucosidase and fucose-containing glycolipids, unknown at the present time, are of interest since fucolipids accumulate in the tissues of patients suffering from 'fucosidosis', an inborn lysosomal disease due to genetic deficiency of α -L-fucosidase (Durand *et al.*, 1968; Dawson & Spranger, 1971).

With the present investigation we started by studying the interactions between α -L-fucosidase and fucolipids, namely a fucose-containing ganglioside with fucose α -glycosidically linked to the 2-position of terminal galactose (Fuc-GM1). A highly purified preparation of α -L-fucosidase, obtained from the hepatopancreas of *Octopus vulgaris*, was employed. In order to differentiate the specific interactions of the enzyme leading to

Abbreviations used: the paper follows the ganglioside nomenclature of Svennerholm (1970) and the IUPAC–IUB recommendations (IUPAC–IUB Commission Biochemical Nomenclature, 1972, 1982); Fuc-GM1, II³NeuAc,IV²Fuc-GgOse₄-Cer; GM1, II³NeuAc-GgOse₄-Cer; GD1a, II³NeuAc,IV³NeuAc-GgOse₄-Cer; GT1b, II³(NeuAc)₂,IV³NeuAc-GgOse₄-Cer; CMC, critical micellar concentration; NeuAc, *N*-acetylneuraminic acid; MUB-fucoside, 4-methylumbelliferyl- α -L-fucopyranoside; MUB, 4-methylumbelliferone; PNP-fucoside, 4-nitrophenyl- α -L-fucopyranoside.

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removal of the fucose moiety from those implicating the ganglioside molecule *per se*, other gangliosides, namely GM1, GD1a and GT1b, structurally similar to Fuc-GM1 but not carrying fucose, were also employed.

Materials and methods

Chemicals and other products

Commercial chemicals were of the highest available grade. Water was doubly distilled in a glass apparatus and used to prepare the different solutions. Crystalline bovine serum albumin and NeuAc were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.); 4-MUB-fucoside, 4-methyl-MUB, PNP-fucoside and 4-nitrophenol were from Koch-Light (Colnbrook, Bucks., U.K.); ultrafiltration apparatus (model 52) and Diaflo UM 10 membranes were from Amicon G.m.b.H. (Witten, Germany); Ultrogel AcA34 was from LKB (Stockholm, Sweden); dialysis tubing was from A. Thomas (Philadelphia, PA, U.S.A.); silica-gel precoated thin-layer plates (HPTLC, Kieselgel 60, 250 μ m thick, 10cm $\times$ 10cm) were from Merck G.m.b.H. (Darmstadt, Germany); preblended liquid-scintillation solution (Instagel) was from Packard (Downers Grove, IL, U.S.A.); surface autoradiography enhancer (En³Hance spray) was from New England Nuclear (Boston, MA, U.S.A.), and films for t.l.c. autoradiography analysis (X-Omat 282) from Kodak (Milan Dealer, Milan, Italy).

Preparation of gangliosides

Gangliosides GM1, GD1a, GT1b were extracted and purified from bovine brain, and Fuc-GM1 from pig brain, as described by Tettamanti *et al.* (1973). Their identification, structure analysis and purity were assessed as described by Sonnino *et al.* (1978). The final purity of all gangliosides, was over 99%. The average M_r of the Fuc-GM1 micelle, determined by a laser-light-scattering technique (Corti *et al.*, 1980), was $500\,000 \pm 28\,000$.

Fuc-GM1 and GM1 were ³H-labelled in the C-3 position of the sphingosine moiety by the method of Ghidoni *et al.* (1981) and purified as described by Gazzotti *et al.* (1984). The radiochemical purity was better than 99% for both compounds, and the specific radioactivity was 0.7 Ci/mmol and 1.5 Ci/mmol for Fuc-GM1 and GM1 respectively.

Preparation of α -L-fucosidase

α -L-Fucosidase (EC 3.2.1.51) from *Octopus hepato-pancreas* was prepared as previously described (D'Aniello *et al.*, 1982). The specific activity of the final purified enzyme, with PNP-fucoside as substrate, was 38.9 units/mg of protein. Its M_r ,

determined by the gel-filtration method, was 70 000–75 000 (D'Aniello *et al.*, 1982).

Preparation of ganglioside samples

Each ganglioside was dissolved in chloroform/methanol (2:1, v/v). A known portion of the mixture, containing the required amount of ganglioside, was transferred into a glass tube and the solvent was completely removed by a gentle flow of N₂. The residue, kept under vacuum over P₂O₅ until processed, was dissolved in a volume of bidistilled water corresponding to 80–90% of the final volume to be used in the individual experiments, left overnight at room temperature (in order to have stable micelles) and then the reaction mixture completed. In most experiments employing Fuc-GM1 a small amount of ³H-labelled compound (about 50 000 d.p.m.) was added to the chloroform/methanol mixture of unlabelled ganglioside and further processed as described above.

α -L-Fucosidase assay

(a) *Fluorimetric method.* Incubation mixtures containing in a final volume of 0.25 ml, 0.1–3 μ g of enzyme (as protein), 0.25 mM-MUB-fucoside (saturating concentration) and 25 mM-citric acid/sodium phosphate buffer, pH 5.6 (optimum value), were incubated at 37°C for 5 min. At the end of the incubation the reaction was stopped by addition of 1.5 ml of 0.2 M-glycine/NaOH mixture, pH 10.5, and fluorescence immediately measured with a Perkin-Elmer model 2000 spectrofluorimeter, using an excitation wavelength of 367 nm and an emission wavelength of 448 nm. The amount of hydrolysed substrate was calculated by means of a standard curve constructed with the use of increasing concentrations of MUB. The control incubation mixtures (blanks) were complete incubation mixtures lacking the enzyme.

(b) *Radiochemical method.* The incubation mixture contained 10–50 μ g (as protein) of α -L-fucosidase, given amounts of unlabelled Fuc-GM1, plus 50 000 d.p.m. of ³H-labelled compound, and 25 mM-citric acid/sodium phosphate buffer, pH 3.4 (optimal value) in a final volume of 0.1 ml, and was incubated at 37°C for a given time. First the ganglioside mixture was prepared (see above), then buffer was added and finally the enzyme. The control incubation mixtures (blanks) were complete incubation mixtures prepared with heat-inactivated enzyme (10 min at 100°C). The enzyme activity was measured by quantification of the ³H-labelled GM1 ganglioside released from [³H]Fuc-GM1. For this purpose the gangliosides contained in the incubation mixture, extracted and purified as described by Tettamanti *et al.* (1973), were separated by t.l.c. under the following experimental conditions: HPTLC silica-gel plates

(length of deposition line 4–5 mm); solvent system propan-1-ol/32% (v/v) NH_3 /water (6:2:1, by vol.); 2 h run at 20°C; detection of the spots (in control experiments) by treating with an Ehrlich spray reagent and heating at 120°C for 15 min (Chigorno *et al.*, 1982) or by autoradiographic analysis; spot identification by co-chromatography with pure standard gangliosides GM1 and Fuc-GM1 (unlabelled and radiolabelled). The distance between Fuc-GM1 and GM1 spots was at least 5 mm. Residual [^3H]Fuc-GM1 and released [^3H]GM1 were quantified by radiochromatoscanning with a Berthold TLC Linear analyser model LB 282, equipped with an Apple II Europlus computer. Portions containing 15000–25000 d.p.m. of radioactivity were spotted and the radiochromatoscanner was programmed in order to accumulate the radioactivity counts from a single plate for 30 min. By knowing (a) the ratio between [^3H]Fuc-GM1 and [^3H]GM1, and (b) the specific concentration of Fuc-GM1 in the starting incubation mixture, the amount of released GM1 could be calculated. The sensitivity and reproducibility of the method were ascertained in control experiments where mixtures of labelled Fuc-GM1 and GM1 were treated under the same experimental conditions and radioscaned. The yield was higher than 92%. The minimum counting for reliable measurement was 50–60, which corresponded to 0.4–0.5% of the total counts recorded. On this basis the sensitivity of the method was at least 0.05 μg of released GM1 starting from 50000 d.p.m. of radioactivity in the incubation mixture.

(c) *Expression of α -L-fucosidase activity.* The activity of α -L-fucosidase was expressed in EC units (μmol of MUB liberated from MUB-fucoside or μmol of GM1 liberated from Fuc-GM1/min at 37°C). K_m and $V_{\max}$ values were determined by the method of Lineweaver & Burk (1934). K_i in inhibition studies was calculated by the Dixon (1953) plot.

Formation and separation of the non-catalytic Fuc-GM1- α -L-fucosidase complex

Fuc-GM1 (0.3–1.5 mg) was dissolved in chloroform/methanol (2:1, v/v) and mixed with 0.15 mCi of [^3H]Fuc-GM1 as tracer; the solvent was completely removed by a gentle flow of N_2 . The dried residue was dissolved in 0.25 ml of 25 mM-citric acid/sodium phosphate buffer, pH 3.4. Known amounts of α -L-fucosidase (50–200 μg , as protein) were then added and the mixture incubated at 37°C for a given time. After the reaction had been stopped by immersion of the incubation mixture in an ice bath, a 0.1 ml portion was analysed for released GM1 (see α -L-fucosidase assay by the radiochemical method), 15 μl was used for the measurement of radioactivity and 0.1 ml

was submitted to chromatography on a column (1 cm $\times$ 200 cm) of Ultrogel AcA34, previously equilibrated with the same above buffer. The following experimental conditions were employed: temperature, 4°C; eluting solvent, 25 mM-citric acid/sodium phosphate buffer, pH 3.4; flow rate, 5 ml/h; 1 ml fractions automatically collected; elution monitored by measurement of protein and radioactivity in each fraction.

The eluted fractions containing both protein and radioactivity were pooled, concentrated (about 1 mg of ganglioside-bound NeuAc/ml) by ultrafiltration on an Amicon apparatus with Diaflo UM-10 membranes, and stored (not longer than 8 h) at 4°C until further processing.

Identification of the product of enzymic hydrolysis of Fuc-GM1

Incubation mixtures containing Fuc-GM1 or Fuc-GM1- α -L-fucosidase complex (prepared as specified above) in a volume of 1 ml were incubated at 37°C for a given time, then cooled and dialysed at 4°C for 12–15 h against 200 vol. of bidistilled water, changed three to four times. The gangliosides contained in the dialysis sac were extracted and purified by the method of Tettamanti *et al.* (1973) and were separated by t.l.c. or column chromatography and then submitted to chemical analysis (Ghidoni *et al.*, 1980).

Other analytical methods

Protein content was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard; ganglioside-bound NeuAc was assayed by the method of Svennerholm (1957), with NeuAc as standard.

Results

Activity of α -L-fucosidase towards MUB-fucoside and micellar Fuc-GM1

The main features of *Octopus* α -L-fucosidase action on MUB-fucoside are shown in Fig 1. At the pH optimum (5.6), the K_m and $V_{\max}$ were 45 μM and 23 μmol of MUB released $\cdot \text{min}^{-1} \cdot \text{mg}$ of protein $^{-1}$ respectively. Under optimal assay conditions the rate of hydrolysis was linear with enzyme quantity up to 3 μg and with incubation time for at least 20 min.

Incubation of α -L-fucosidase with Fuc-GM1, at concentrations largely above the CMC, was followed by liberation of GM1 with maximal activity at pH 3.4 (Fig. 2). As shown in Fig. 3, the $v/[S]$ and $v/[E]$ relationships were biphasic and sigmoidal-shaped in the first phase of the curve, with transition points dependent on the enzyme/ganglioside ratio. The enzyme activity towards Fuc-GM1 was linear with time for 8–10 min.

Fig. 1. Kinetics of *Octopus* α -L-fucosidase action on a low- M_r substrate (MUB-fucoside). See the Materials and methods section for assay conditions.

The anomalous behaviour of the enzyme kinetics suggested (Gatt & Bartfai, 1977; Venerando *et al.*, 1985) the possible formation of inactive complex(es) between α -L-fucosidase and ganglioside during incubation. This possibility was inspected by preincubating the enzyme with 0.1 mM-ganglioside (Fuc-GM1, GM1, GD1a or GT1b) at different pH values for 30 min, followed by assay of the enzyme in the presence of MUB-fucoside. As shown in Fig. 4, ganglioside treatment had no effect on the enzyme activity at pH values over 5.0 and started inhibiting below pH 5.0, the inhibition becoming complete at pH values lower than 3.7. The inhibitory effect of ganglioside treatment at low pH values was the same regardless of the ganglioside species used. The inhibition by treatment with gangliosides (Fuc-GM1, GM1, GD1a, GT1b) was directly dependent on ganglioside concentration (100% inhibition at 0.1 mM) and preincubation time (100% inhibition with 0.1 mM-ganglioside after 30 min.) (Fig. 5) and was of a non-competitive nature (Fig. 6).

Formation and separation of the non-catalytic Fuc-GM1- α -L-fucosidase complex

The formation and separation of inactive or less-active enzyme-ganglioside complex(es) was accurately investigated. It was preliminarily observed that α -L-fucosidase, when loaded on the Ultrogel AcA34 column (see the Materials and methods section), could not be eluted even after prolonged

washing with 25 mM-citric acid/sodium phosphate buffer, pH 3.4. It was proved that the enzyme remained linked in a fully active form on the top layer of the column. When a mixture of Fuc-GM1 (in radioactive form) and α -L-fucosidase in 25 mM-citric acid/sodium phosphate buffer, pH 3.4, was submitted to column chromatography on Ultrogel AcA34 at zero time of incubation, the only eluted peak was that of radioactivity corresponding to Fuc-GM1 micelles (Fig. 7a). After incubation at 37°C for different times, two peaks of radioactivity appeared. One had the same elution volume of Fuc-GM1 micelles, although smaller than that recorded at zero time; the second, which contained ganglioside and protein, was eluted closer to the void volume of the column, indicating the presence of material of increased M_r (Fig. 7b). By prolonging the time of incubation the peak corresponding to the ganglioside micelles became smaller, with a concomitant increase in the peak containing both radioactivity and protein. Furthermore, by enhancing the enzyme/ganglioside ratio in the starting mixture, the peak corresponding to ganglioside micelles tended to disappear, whereas that carrying both radioactivity and protein increased (Fig. 7c). This peak likely corresponded to a Fuc-GM1- α -L-fucosidase complex formed during incubation. The presence of any 'free' form of the enzyme could be ascertained by assaying the α -L-fucosidase activity remaining in the top layer of the column after exhaustive washing. By using 1 mM-Fuc-

Fig. 2. Hydrolysis of Fuc-GM1 by the action of *Octopus* α -L-fucosidase

A standard incubation mixture, and the corresponding blank, each containing 35 μ g of α -L-fucosidase, as protein, and 3.5 mM-Fuc-GM1, containing 50000 d.p.m. of radioactivity, was incubated at 37°C for 30 min, then stopped by tetrahydrofuran treatment. Gangliosides were extracted from the incubation and blank mixtures, chromatographed and submitted to radiochromatoscanning as described in the Materials and methods section. (a) Radiochromatoscanning of the blank mixture; (b) radiochromatoscanning of the incubated mixture; (c) t.l.c. of standard gangliosides Fuc-GM1 and GM1.

GM1 and 0.5 mg (as protein) of α -L-fucosidase/ml and at least 60 min incubation at 37°C, 100% of the protein loaded on the column was recovered in the peak of the complex, with complete disappearance of any α -L-fucosidase activity in the top layer of the column.

The observed behaviour was quite reproducible provided that new columns were used for each experiment. This renewal was necessary owing to (a) the free form of α -L-fucosidase being retained on the resin, and (b) the behaviour of radioactivity during column chromatography. In fact only 85–90% of the loaded radioactivity could be eluted from the column, whereas the remaining

Fig. 3. Apparent kinetics of *Octopus* α -L-fucosidase action on ganglioside Fuc-GM1

The enzyme was directly incubated in the presence of Fuc-GM1 in micellar form. (a) $v/[S]$ relationships studied with two different amounts of enzyme in the incubation mixture [35 (●) or 10 (▲) μ g of α -L-fucosidase]; (b) $v/[E]$ relationship studied in the presence of 3.5 mM-Fuc-GM1; (c) time course of the enzymic hydrolysis of Fuc-GM1 studied by using 35 μ g of enzyme and 3.5 mM-ganglioside. Fuc-GM1 molarity is expressed as bound NeuAc. For details, see the Materials and methods section.

portion adhered to the gel materials, probably in the form of monomeric gangliosides (Tomasi *et al.*, 1980).

Under the conditions where the enzyme was completely transported into a complex, the release of GM1 was very modest owing to the transient presence of free (and therefore working) enzyme during the formation of the complex. In contrast, under conditions where the enzyme was partly present in the free form at the end of preincubation, the extent of GM1 release was higher and depended on the amount of free enzyme and Fuc-GM1- α -L-fucosidase complex.

Partial characterization of isolated Fuc-GM1- α -L-fucosidase complex

Mixtures containing 1 mM-Fuc-GM1 (with radioactive compound added), α -L-fucosidase

Fig. 4. Inhibition of *Octopus* α-L-fucosidase after preincubation at different pH values in the presence of gangliosides Fuc-GM1 (●) or GM1 (○).

The enzyme (3.5 μg) was preincubated in the presence of 0.1 mM-ganglioside for 1 h at 37°C and then assayed with MUB-fucoside as substrate under the optimized conditions for the fluorimetric method described in the Materials and methods section. Similar results were obtained with gangliosides GD1a and GT1b.

Fig. 5. Effect of ganglioside concentration and of preincubation time on the inhibition of *Octopus* α-L-fucosidase by gangliosides.

The enzyme (3.5 μg) was preincubated at pH 3.4 and at 37°C (a) at different concentrations of ganglioside [GM1 (○), Fuc-GM1 (□), GD1a (●) or GT1b (△)] for 40 min or (b) at fixed ganglioside concentration (0.1 mM) for different times and then assayed with MUB-fucoside as substrate under the optimized conditions of the fluorimetric method. Ganglioside concentration is expressed as bound NeuAc. For details, see the Materials and methods section.

Fig. 6. Inhibition of *Octopus* α-L-fucosidase by preincubation with gangliosides GM1 (○) and Fuc-GM1 (●) expressed according to the Lineweaver-Burk (1934) plot.

The enzyme (3.5 μg) was preincubated at pH 3.4 and at 37°C with different concentrations of ganglioside and then incubated with increasing concentrations of MUB-fucoside under the optimized conditions of the fluorimetric assay. Similar results were obtained with gangliosides GD1a and GT1b.

(0.5 mg/ml, as protein) and 25 mM-citric acid/sodium phosphate buffer, pH 3.4, were incubated at 37°C for 60 min. Under these conditions the enzyme was completely converted into the complex (see above). The incubated mixture was chromatographed on an Ultrogel AcA34 column and the eluted fractions corresponding to the complex were pooled and concentrated at 4°C to 1.5–2 ml by ultrafiltration. The complex, when submitted to rechromatography under the same conditions, gave a peak corresponding to the original one. On storage of the complex at 37°C (for 8 h), no appreciable generation of free enzyme and free ganglioside was observed. Moreover, incubation of the complex with various amounts of unlabelled Fuc-GM1 did not cause any release of radioactivity. Addition of bovine serum albumin was not able to release radioactivity from the complex. The content of ganglioside (calculated on the basis of bound NeuAc) relative to that of protein (w/w) was 4.0 mg/mg of protein. The ganglioside contained in the complex was Fuc-GM1 (about 93%) and GM1 (about 7%), the latter formed during the formation of the complex.

Fuc-GM1-α-L-fucosidase complex, incubated at 37°C in 25 mM-citric acid/sodium phosphate buffer, pH 3.4, for up to 14 h, did not release GM1. The same complex, incubated in the presence of various amounts of MUB-fucose (at pH 5.6) or of Fuc-GM1 (at pH 3.4), did not display any hydrolytic activity. Conversely, treatment of the complex with Triton X-100 (0.1%, w/w, final concn.) for 30 min at 37°C was followed by substantial recovery of α-L-fucosidase activity, measured as

Fig. 7. Separation of Fuc-GM1- α -L-fucosidase complex formed under the conditions used for the enzyme assay. Column chromatography on Ultrogel AcA34 was carried out as described in the Materials and methods section. Conditions: enzyme, 60 μ g (a,b,c,e) or 20 μ g (d); Fuc-GM1 (with the addition of 50000 d.p.m. of 3 H-labelled compound), 1.5 mM; incubation time, various, from 0 to 60 min (a,b,c), or fixed, 60 min (d,e). The elution of ganglioside was monitored by measurement of radioactivity and that of protein by the Lowry method (Lowry *et al.*, 1951). For details of the conditions for complex-formation and separation, see the Materials and methods section. (a), (b) and (c), effect of incubation time on the formation of the complex; (d) and (e), effect of the enzyme/ganglioside ratio on the formation of the complex.

MUB-fucose. Inactivation of α -L-fucosidase by complex-formation was also obtained with gangliosides GM1, GD1a and GT1b.

Action of α -L-fucosidase on Fuc-GM1- α -L-fucosidase complex

As shown in Fig. 8, α -L-fucosidase affected the complex and released GM1 (optimal pH, 3.4). The

Fig. 8. Kinetics of *Octopus* α -L-fucosidase activity towards the Fuc-GM1- α -L-fucosidase complex

The complex was prepared and separated as described in the text and submitted to the action of free α -L-fucosidase under the optimized conditions of the radiochemical method. (a) $v/[S]$ experiments carried out with 35 μ g of free enzyme; (b): $v/[E]$ experiments carried out at 1 mM-Fuc-GM1- α -L-fucosidase complex. Substrate molarity is expressed as ganglioside NeuAc in the complex.

enzyme activity was linear with respect to enzyme-protein concentration up to 40 μ g/assay and with incubation time up to 60 min. The $v/[S]$ relationship obeyed regular hyperbolic kinetics and the K_m and V_{max} values were 0.12 mM and 2.4 μ mol of GM1 released $\cdot$ min $^{-1}$ $\cdot$ mg of protein $^{-1}$ respectively. The data shown in Fig. 9 indicate that, on incubation of the complex in the presence of added α -L-fucosidase, Fuc-GM1 was transformed into a ganglioside that co-chromatographed with authentic GM1. The ganglioside enzymically produced from Fuc-GM1- α -L-fucosidase complex was isolated and submitted to chemical analysis. The results obtained were consistent with the composition and structure of ganglioside GM1.

Discussion

The present investigation provides the first detailed information on the action of an α -L-fucosidase, namely the enzyme from *Octopus*

Fig. 9. T.l.c. separation and autoradiographic detection of the hydrolysis product of Fuc-GM1- α -L-fucosidase complex by the action of free α -L-fucosidase

The incubation mixture contained Fuc-GM1- α -L-fucosidase complex and free α -L-fucosidase. In the corresponding blank mixture, free α -L-fucosidase was heat-inactivated before incubation. Both the incubation mixture and the blank mixture were submitted to 1 h incubation at 37°C. For details see the Materials and methods section. 1, t.l.c. with colorimetric detection of the ganglioside spots; 2, t.l.c. with autoradiographic detection of the ganglioside spots. (a) standard Fuc-GM1; (b) 'blank' mixture; (c) incubation mixture; (d) standard GM1. The faint band of GM1 present in the blank mixture represents the small amount of the GM1 formed from Fuc-GM1 during the formation of the Fuc-GM1- α -L-fucosidase complex.

hepatopancreas, on fucose-containing gangliosides, particularly Fuc-GM1. The kinetics of *Octopus* α -L-fucosidase action on a low- M_r substrate (MUB-fucoside) appeared quite different from those exhibited on Fuc-GM1. In the former case (see also D'Aniello *et al.*, 1982) the $v/[S]$ and $v/[E]$ relationships were hyperbolic, reflecting regular enzyme-substrate interactions; in the latter case they were sigmoidal-shaped, possibly indicating (Gatt & Bartfai, 1977) the occurrence of enzyme-ganglioside interactions different from, and additional to, those which implicate the catalytic site of the enzyme. This variety of interactions might likely result from the high reactivity of ganglioside micelles, which are known to be formed in aqueous solution within a wide range of ganglioside concentrations, from 10–1 nM to 10 mM (Curatolo *et al.*, 1977; Formisano *et al.*, 1979).

Incubation of pure α -L-fucosidase with Fuc-GM1 was followed by the formation of a ganglioside-protein complex that was not catalytically active. Since the ganglioside concentration was always over 10 μ M, Fuc-GM1 was present and

interacting mainly as micelle. Moreover, α -L-fucosidase also formed complexes and underwent inactivation with gangliosides GM1, GD1a and GT1b, which are not substrates for the enzyme. Thus the complex is a combination of the enzyme protein with micelles of gangliosides, the combination resulting in complete enzyme inactivation.

The average M_r of a Fuc-GM1 micelle was 500 000, that of the enzyme 70 000–75 000 (D'Aniello *et al.*, 1982), and that of the complex almost certainly in excess of 500 000. In addition, the ganglioside/protein ratio in the complex was about 4:1 (w/w). These data suggest that the complex resulted from association of one ganglioside micelle and two enzyme molecules, a situation analogous to the complexes formed by bovine serum albumin and ganglioside GM1 (Tomasi *et al.*, 1980). The complex was easily affected by free α -L-fucosidase, indicating that the carbohydrate portion of Fuc-GM1 was exposed on the surface. Moreover, the complex did not originate or exchange the starting components on prolonged storage or treatment with ganglioside micelles, indicating that the formation of the complex was an apparently irreversible phenomenon and the complex quite stable. However, treatment of the complex with the non-ionic detergent Triton X-100 resulted in re-appearance of enzyme activity towards MUB-fucoside. Thus the 'inactivity' of the complex-bound α -L-fucosidase would seem to be the consequence of non-accessibility of the enzyme to added substrate. A quite similar behaviour has been recently described (Venerando *et al.*, 1985) for the cytosolic sialidase from pig brain acting on gangliosides, suggesting that micellar gangliosides might be capable of inactivating some enzymes in a potentially reversible way.

All this explains why the kinetics of α -L-fucosidase action on micellar Fuc-GM1 appeared anomalous. In fact during incubation the enzyme formed a complex with Fuc-GM1, in which form the enzyme was inactive; concurrently the remain-ign free form of enzyme could attack the newly formed Fuc-GM1-enzyme complex. Therefore the extent of enzyme hydrolysis was not dependent on the amount of enzyme initially present, nor on the starting ganglioside concentration, but on the amounts of 'free' enzyme and of Fuc-GM1-enzyme complex, which varied under the different experimental conditions. Particularly because the ratio of the 'free' and the 'complex-bound' form of the enzyme was dependent on the actual ganglioside/enzyme ratio, and the bound form was predominant at high ratios, the $v/[E]$ relationship appeared sigmoidal-shaped. This line of interpretation is further supported by the observation that α -L-fucosidase, when acting on the Fuc-GM1-enzyme complex, displayed a kinetic profile

obeying the classic Michaelis-Menten enzyme-substrate interaction.

In conclusion, the present work illustrates a new aspect of the peculiarity of ganglioside-enzyme interactions, that is, reversible inactivation of the enzyme after binding to ganglioside micelles. Moreover, it stresses the necessity of studying ganglioside enzymology by experimental approaches that are different from the conventional ones.

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