THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 279, No. 5, Issue of January 30, pp. 3169–3179, 2004

Printed in U.S.A.

Properties of Recombinant Human Cytosolic Sialidase HsNEU2

THE ENZYME HYDROLYZES MONOMERICALLY DISPERSED GM1 GANGLIOSIDE MOLECULES*

Received for publication, July 31, 2003, and in revised form, November 12, 2003
Published, JBC Papers in Press, November 12, 2003, DOI 10.1074/jbc.M308381200

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Recombinant human cytosolic sialidase (HsNEU2), expressed in *Escherichia coli*, was purified to homogeneity, and its substrate specificity was studied. HsNEU2 hydrolyzed 4-methylumbelliferyl α-NeuAc, α2→3 sialyllactose, glycoproteins (fetuin, α-acid glycoprotein, transferrin, and bovine submaxillary gland mucin), micellar gangliosides GD1a, GD1b, GT1b, and α2→3 paragloboside, and vesicular GM3. α2→6 sialyllactose, colominic acid, GM1 oligosaccharide, whereas micellar GM2 and GM1 were resistant. The optimal pH was 5.6, kinetics Michaelis-Menten type, V_{max} varying from 250 IU/mg protein (GD1a) to 0.7 IU/mg protein (α_1-acid glycoprotein), and K_m in the millimolar range. HsNEU2 was activated by detergents (Triton X-100) only with gangliosidic substrates; the change of GM3 from vesicular to mixed micellar aggregation led to a 8.5-fold V_{max} increase. HsNEU2 acted on gangliosides (GD1a, GM1, and GM2) at nanomolar concentrations. With these dispersions (studied in detailed on GM1), where monomers are bound to the tube wall or dilutedly associated (1:2000, mol/mol) to Triton X-100 micelles, the V_{max} values were 25 and 72 μIU/mg protein, and K_m was 10 and 15 × 10^{-9} M, respectively. Remarkably, GM1 and GM2 were recognized only as monomers. HsNEU2 worked at pH 7.0 with an efficiency (compared with that at pH 5.6) ranging from 4% (on GD1a) to 64% (on α_1-acid glycoprotein), from 7% (on GD1a) to 45% (on GM3) in the presence of Triton X-100, and from 30 to 40% on GM1 monomeric dispersion. These results show that HsNEU2 differentially recognizes the type of sialosyl linkage, the aglycone part of the substrate, and the supramolecular organization (monomer/micelle/vesicle) of gangliosides. The last ability might be relevant in sialidase interactions with gangliosides under physiological conditions.

Sialidases (*N*-acylneuraminosylglycohydrolases; EC 3.2.1.18) constitute a group of enzymes that hydrolytically remove sialic acid residues from sialoglycoconjugates. The wide occurrence of these enzymes from viruses and bacteria to vertebrates (1) highlights their importance in regulating the sialic acid content and turnover of sialoglycoconjugates, molecules that are fundamental to several cellular processes (2). Due to their easier availability, viral and bacterial sialidases have been extensively studied at the molecular level, and the three-dimensional structure of several of them has been established (3–5). Despite an amino acid sequence homology of only 35%, viral and bacterial sialidases share a similar molecular architecture with the protein chains folded into six-sided β-sheet barrels and the active site constituted by almost the same amino acids and organized in a similar manner (6).

The molecular features of mammalian sialidases, including the human enzymes, remained long obscure, since factors like subcellular localization (cytosol, plasma membrane, and lysosomes), low cellular content, and the labile nature of the purified enzymes prevented workers from obtaining sufficient amounts of pure and active enzyme for detailed studies (7, 8). Beginning in 1993, several mammalian sialidases started being cloned from the corresponding cDNAs, prompting a great acceleration of the studies aimed at ascertaining their structural and kinetic properties (9, 10). Knowledge of the amino acid sequences of these sialidases provided insight into the following: (i) the differences between the lysosomal (NEU1) (11–13), cytosolic (NEU2) (14, 15), and plasma membrane-associated (NEU3) sialidases (16, 17), which are unequivocally different enzymes, and (ii) the structural homologies indicating that they belong, unequivocally too, to a unique family of proteins. Of course, a great advance provided by expressing mammalian sialidases in suitable host cells was to obtain pure proteins in adequate amounts and sufficient stability to perform systematic studies.

Issues concerning mammalian sialidases that still require deeper understanding are their relationships with the putative natural substrates, particularly sialoglycolipids (gangliosides) and sialoglycoproteins and the conditions of their action under physiological conditions, since most of them feature *in vitro* an acidic optimal pH (1, 2, 18, 19). It is known that sialidases occur cellularly at different sites (plasma membrane, lysosomes, and cytosol), and sialoglycoconjugates occur primarily at the level of the plasma and Golgi's stack membranes (18, 19) or inside the lysosomes, where their complete degradation occurs (2, 18). Another possible difference can reside in the topology of membrane location (sidedness) of sialidases and sialoglycoconjugates. It is also known that (some) gangliosides can be highly enriched in lipid rafts on the plasma membrane together with other lipid components and specialized proteins (20). These

* This work was supported in part by 60% MURST funds and MURST Cofin 2002 (Molecular Biology and Functional Biochemistry of Human Sialidases) (to G. T. and E. M.) and MURST Cofin 2002 (to A. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) NM005383.

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structures move within the fluid bilayer and may function as mobile platforms able to transduce signals from the outside to the interior of the cell (21). Of course, gangliosides are also present in a much more "diluted" way in the areas of the membrane surrounding rafts. Therefore, gangliosides occur as "patched" or "dispersed" molecules (monomeric dispersions), this constituting a further possible matter of differential recognition by sialidases. With regard to the physiological environment, the only one having an acidic pH is the lysosomal matrix, where the lysosomal sialidase works. However, both the plasma membrane-bound and the cytosolic sialidases, besides the lysosomal one, display an acidic pH optimum (1, 2). On these premises and with the aim to provide a deeper insight into sialidase action, we (i) purified to homogeneity the recombinant form of the human cytosolic sialidase NEU2 (HsNEU2), produced by *Escherichia coli* cells, (ii) carried out a basic biochemical characterization of the enzyme, (iii) studied the specificity of the enzyme action on different sialoglycoconjugates, with particular attention to the ability of the enzyme to differentially recognize the aggregated (micellar) form and monomeric dispersion of the ganglioside substrates, and (iv) verified the ability of the enzyme to act at neutral pH.

EXPERIMENTAL PROCEDURES

Commercial chemicals were of the highest grade available. *N*-Acetyl-D-neuraminic acid (NeuAc), 4-methylumbelliferyl α-NeuAc (MU-NeuAc),[1] and 4-methylumbelliferone, the *N*-glycosylated glycoproteins human fetuin, human α_1-acid glycoprotein, human transferrin, and *O*-glycosylated glycoprotein mucin from bovine submaxillary glands, α2→3 sialyllactose sodium salt (purity, better than 98%), α2→6 sialyllactose (purity better than 98%), crystalline bovine serum albumin, sialidases (from *Arthrobacter ureafaciens*, *Salmonella typhimurium* (recombinant), *Vibrio cholerae*, and *Clostridium perfringens*), Triton X-100, CHAPS, octyl glucoside, and sodium deoxycholate were provided by Sigma; [^{3}H]borohydride (6500 Ci/mol^{-1}) was provided by Amersham Biosciences; DEAE-Sephadex A-25 was from Amersham Biosciences; Dowex 2 × 8 resin (200–400-mesh), prepared in acetate form (22), was from Bio-Rad; cationic and anionic exchange columns (Mono S HR 5/5 and Mono Q HR5/5), glutathione (GSH) 4B-Sepharose, and thrombin were from Amersham Biosciences; ultrafiltration apparatus Mod.52 and Diaflo membranes (cut-off 10 kDa) were from Amicon (Witten, Germany); precoated silica gel high performance thin layer chromatography (HPTLC) plates (Kiesel gel 60, 250-μm thickness, 10 × 20 cm) and precoated cellulose thin layer plates (cellulose: 100-μm thickness, 10 × 20 cm) were from Merck; and water was doubly distilled in a glass apparatus and used to prepare the different solutions. The oligosaccharide from ganglioside GM1 was prepared according to Wiegandt and Bucking (23). Standard molecular biology techniques were performed as described by Sambrook and Russell (24). DNA restriction and modifying enzymes were from Roche Applied Science unless indicated otherwise.

Expression and Purification of Recombinant HsNEU2

The entire coding region of *HsNEU2* was obtained by PCR amplification using the isolated genomic clone HG7 as template as previously described (15). Briefly, the DNA sequence from the Met to the Gln in position 67 (exon 1) was amplified using *NEU2*-Nt (5′-CGTAGATCTATGGCGTCCCTTCCTGTCCTG-3′) with a 5′-BamHI restriction site and a 5′-phosphorylated antisense primer (NEU2–67R; 5′-CTGAACCTGGTGGGTGGGTGC-3′). The DNA sequence from the Trp in position 68 to the stop codon (exon 2) was amplified using (*NEU2*-68F; 5′-TGGCAAGCTCAGGAGGTGGTG-3′) and *NEU2*-Ct (5′-CATGAATCCTCACTGAGGCAGGTACTG-3′) with a 5′-EcoRI restriction site. The two PCR products, obtained using cloned *Pfu* polymerase (Stratagene, La Jolla, CA), were ligated using T4 ligase, and the resulting fragment was subcloned into BamHI-EcoRI sites of pGEX-2T expression vector (Amersham Biosciences). The recombinant plasmid, pGEX-2T-*NEU2*, was completely sequenced using both vector- and gene-specific primers. *E. coli* DH5 α cells were transformed with the recombinant plasmid pGEX-2T-*NEU2* or the expression vector pGEX-2T alone, and the transformed cells were grown in LB-ampicillin medium at 37 °C to midlog phase before the addition of isopropyl-α-D-thiogalactopyranoside (Sigma) to 0.05 mM. After overnight growth at room temperature, cells were harvested by centrifugation and suspended in 50 volumes of phosphate-buffered saline (PBS). Cells lysed by sonication and supernatants obtained after centrifugation at 12,000 × *g* for 10 min were assayed for sialidase activity using MU-NeuAc as substrate. Purification of glutathione *S*-transferase (GST)-HsNEU2 fusion protein was carried out by affinity chromatography on a GSH-Sepharose 4B column (using the GST portion of the chimeric protein for affinity binding) according to the manufacturer's instructions (GST Gene Fusion System; Amersham Biosciences), with minor modifications. After an additional washing with PBS plus 0.5 M NaCl, cleavage of GST from the GST-HsNEU2 fusion protein bound to the matrix was carried out adding 0.5 IU of thrombin/ml in PBS and incubating the column for 2–3 h at room temperature. The resulting eluted enzyme was further purified by two successive high performance chromatographic steps. The first was on a MONO Q HR5/5 column equilibrated with 20 mM Tris/HCl, pH 7.3. The enzyme activity was eluted in the column flow-through and the collected fractions carrying the enzyme were dialyzed against four changes of 50 volumes of 50 mM MES buffer, pH 6.7, at 4 °C, concentrated under vacuum to about 2 ml in a rotating evaporator and applied to a MONO S HR 5/5 column equilibrated in the same buffer. The enzyme was eluted using a linear gradient of 50 mM MES buffer, pH 6.7, containing 1 M NaCl. The two chromatographic steps were carried out at room temperature with an elution rate of 1 ml/min and using an FPLC apparatus (Amersham Biosciences). Protein samples of *E. coli* crude lysate and of the different fractions obtained along the purification procedure were assayed for sialidase activity and protein content and monitored for purity by reducing SDS-PAGE (25) with GELCODE Blue Stain protein detection reagent (Pierce). The purity of the final enzyme preparation was assessed also by isoelectrofocusing according to Righetti (26).

Preparation of Cold and ^{3}H-Labeled Gangliosides and Neutral Glycolipids

Gangliosides GM1, GM2, GD1a, and GD1b from calf brain (27), GM3 (carrying NeuAc) from human spleen (28), and α2→3 sialosylparagloboside (α2→3SPG) from human erythrocytes (29) were prepared and structurally analyzed as previously described (30). Asialo-GM1 (GA1) was prepared according to Ghidoni *et al.* (31). GM1, GM2, and GD1a were ^{3}H-labeled at C-3 of the sphingosine (Sph) moiety (32), and the *erythro* forms, separated and purified (33), were employed. GM3 was ^{3}H-labeled at the level of the *N*-acetyl group of sialic acid ([*NeuAc*-^{3}H]GM3) according to Chigorno *et al.* (34). GA1 ^{3}H-radiolabeled at the level of Sph was prepared according to Ref. 35. The radiochemical purity of [*Sph*-^{3}H]GM1, [*Sph*-^{3}H]GM2, [*Sph*-^{3}H]GD1a, [*NeuAc*-^{3}H]GM3, and [*Sph*-^{3}H]GA1 was better than 99%, and the specific radioactivity was 1.63, 0.96, 1.85, 0.60, and 1.58 Ci mmol^{-1}, respectively. Radiolabeled gangliosides were stored at −20 °C in *n*-propyl alcohol/water (4:1, v/v), and maintenance of radiochemical purity was assessed before use.

Preparation of Homogeneous Micelles (or Vesicles) of Gangliosides or of Mixed Micelles of Gangliosides and Detergents

It is known that gangliosides form micelles in water solution. Their critical micellar concentration (CMC) is in the range 10^{-8} to 10^{-7} M, depending on the ganglioside (36). Therefore, at concentrations above the CMC (*e.g.* in the millimolar range), they are present essentially as micelles, whereas below the CMC (*e.g.* in the nanomolar range) they are present only as monomers. The only exception is ganglioside GM3 that undergoes vesicular aggregation in aqueous solution (36). Homogenous micelles of gangliosides α2→3SPG, GM1, GM2, GD1a, GD1b, and GT1b and homogenous vesicles of GM3 (with the addition of 0.15 μCi of the ^{3}H-labeled form in the cases of GM1, GM2, GD1a, and GM3) were prepared as follows. Each ganglioside was dissolved in chloroform/

[1] The abbreviations used are: MU-NeuAc, 4-methylumbelliferyl α-NeuAc; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; GST, glutathione *S*-transferase; PBS, phosphate-buffered saline; MES, 4-morpholineethanesulfonic acid; FPLC, fast protein liquid chromatography; α2→3SPG, α2→3 sialosylparagloboside; CMC, critical micellar concentration; HPTLC, high performance thin layer chromatography; GM2-AP, GM2 activator protein. The ganglioside nomenclature proposed by Svennerholm was followed (Svennerholm, L. (1980) *Adv. Exp. Med. Biol.* **125,** 11). *NEU* is the official human gene symbol approved by HUGO Gene Nomenclature Committee. In order to avoid misinterpretation, the NEU symbol indicates the gene product (*i.e.* sialidase); NEU2 is the symbol for cytosolic sialidase; the two-letter code preceding NEU2 indicates the species of origin (*e.g.* Hs indicates *Homo sapiens*).

methanol (2:1, v/v), and a known volume of this solution, containing the desired amount of ganglioside, was transferred into an Eppendorf tube, the solvent being then completely removed by nitrogen fluxing. The residue was kept for several hours under vacuum over P_2O_5 and then dissolved with 60 μl of redistilled water. The mixture was left to stand overnight at room temperature before use (37). Mixed micelles of gangliosides and detergent (Triton X-100) were prepared as follows. Detergent (at millimolar concentrations) was dissolved in chloroform/methanol (2:1, v/v), and a known volume of this solution was pipetted into an Eppendorf tube and mixed with a sample of the starting ganglioside solution in order to have the desired proportion of the two components. The solvent was completely removed by nitrogen fluxing, and the resulting residue was further dried, dissolved in 60 μl of redistilled water, and allowed to equilibrate at room temperature, as described above. At the end, each mixture was buffered and used for sialidase assay in a final volume of 100 μl.

Preparation of Monomeric Dispersions of Gangliosides

Dispersions on Tube Walls

A known volume of ganglioside solution in *n*-propyl alcohol/water (4:1, v/v), containing the desired amount (0.1–6.4 pmol) of ^{3}H-labeled GM2, GM1, or GD1a was transferred into an Eppendorf tube, and the solvent was completely removed by nitrogen fluxing. The residue was kept for several hours under vacuum over P_2O_5 and then dissolved with 60 μl of redistilled water and allowed to equilibrate at room temperature. At the end, the mixture was buffered and used for sialidase assays, in a final volume of 100 μl. Under these conditions, the ganglioside molecules are known (36) to adhere, at least partially, to the tube surface. In order to establish the distribution of ganglioside, as well as the sialidase reaction product(s), between the aqueous solution and the tube surface, the aqueous assay mixture (before and after incubation with sialidase) was transferred with a Pasteur pipette into a tube, the pipette being washed several times with methanol, and the washings were added to the pipetted incubation mixture. The resulting mixture, containing the glycolipid in free solution, was frozen and lyophilized. The residue was resuspended with 5 ml of emulsifier (Ultima Gold; Packard), and the radioactivity was determined with a liquid scintillation counter (Packard Tri-Carb 460). The starting Eppendorf tube, carrying the wall-bound ganglioside, was added with 0.1 ml of chloroform/methanol (2:1, v/v) and mixed, and the radioactivity was measured as described above.

The conditions providing passage of monomeric dispersions of glycolipid from the tube wall to the aqueous medium were worked out with preliminary experiments using [*Sph*-^{3}H]GM1 and Triton X-100. The experimental approaches were as follows. (i) 0.1–2 pmol of [*Sph*-^{3}H]GM1 in *n*-propyl alcohol/water (2:1, v/v) and different amounts of Triton X-100 (0.4–40 nmol) in chloroform/methanol (2:1, v/v) were transferred into an Eppendorf tube, and the solvent was completely removed by nitrogen fluxing. The resulting residue was kept for several hours under vacuum over P_2O_5 and finally dissolved with 60 μl of redistilled water and allowed to equilibrate at room temperature. The mixture was then added with buffer (at the optimal sialidase pH 5.6 or at pH 7.0) and enzyme (final volume 100 μl) and used for sialidase assays. The distribution of GM1 and sialidase reaction product in the aqueous solution and tube wall was determined as described above. (ii) 40 nmol of Triton X-100 in chloroform/methanol (2:1, v/v) and different amounts of [*Sph*-^{3}H]GM1 (0.9–83 pmol) in *n*-propyl alcohol/water (4:1, v/v) were transferred in an Eppendorf tube, and the solvent was completely removed by nitrogen fluxing; the successive procedural steps were the same as described above.

Dispersions on the Surface of Triton X-100 Micelles

On the basis of the indications provided by the experimental approaches described above, complete dispersion of [*Sph*-^{3}H]GM1 on the surface of Triton X-100 micelles was obtained mixing radiolabeled GM1 and Triton X-100 in a molar ratio of 1:2000. On this basis, the proper amount of [*Sph*-^{3}H]GM1 in *n*-propyl alcohol/water (4:1, v/v) and a 2000-fold (in molar terms) amount of Triton X-100 in chloroform/methanol (2:1, v/v) were pipetted into an Eppendorf tube, and the solvent was completely removed by nitrogen fluxing; the subsequent steps were the same described above.

Sialidase Assay

The activity of HsNEU2 sialidase was determined essentially as described by Venerando *et al.* (38). The reaction mixtures contained the established amounts of HsNEU2 and substrate (see below), 50 mM sodium citrate/phosphate buffer at the optimum pH (established to be 5.6 and at pH 7.0) in a final volume of 100 μl. The control mixtures (blanks) were carried out using heat-inactivated (5 min in boiling water) recombinant HsNEU2. K_m and apparent V_{max} values were determined by the method of Lineweaver and Burk. The sialidase activity was expressed as IU (μmol of liberated NeuAc/min at 37 °C). The results are always the means of two or three separate experiments carried out in triplicate. The influence on sialidase activity of the following detergents was studied: Triton X-100, CHAPS, octyl glucoside, and sodium deoxycholate. The following assay methods were employed.

Fluorimetric Method

The reaction mixtures (100 μl) set up with an appropriate concentration of MU-NeuAc, in the presence or absence of 20 μg of bovine serum albumin and of different amounts of a detergent (when used) were incubated at 37 °C with recombinant HsNEU2 (0.02 μg at pH 5.6; 0.15 μg at pH 7.0) for 10 min (pH 5.6) or 20 min (pH 7.0). Reactions were stopped by the addition of 1.5 ml of 0.2 M glycine/NaOH, pH 10.2. Fluorescence emission was measured on a Jasco FP-770 fluorometer with excitation at 365 nm and emission at 445 nm, using 4-methylumbelliferone to set up a calibration curve. A fluorescence of 0.100 corresponded to 0.16 nmol of released 4-methylumbelliferone.

Colorimetric Method

The reaction mixtures (100 μl) were set up with appropriate concentrations of α2→3 (or α2→6) sialyllactose, colominic acid, sialoglycoprotein (human fetuin, human α_1-acid glycoprotein, human transferrin, and mucin from bovine submaxillary glands), or gangliosides α2→3SPG, GD1b, and GT1b (the ganglioside concentration was in the millimolar range). After incubation at 37 °C with recombinant HsNEU2 (0.2 μg at pH 5.6; 1.5 μg at pH 7.0) up to 90 min (depending on the substrate), released sialic acid was measured by the chromatographic microprocedure of Caimi *et al.* (39).

Radiochemical Methods

Ganglioside Substrates Used as Micellar Aggregates—The incubation mixtures (100 μl), containing different concentrations (in the millimolar range) of micellar ^{3}H-labeled ganglioside GM1, GM2, or GD1a or vesicular GM3 (each carrying 0.15 μCi of radioactivity) and in the presence or absence of different amounts of detergent, were incubated at 37 °C with recombinant HsNEU2 (0.02 μg at pH 5.6; 0.15 μg at pH 7.0) for up to 10 min (pH 5.6) or 30 min (pH 7.0) or up to 60 min in the case of GM1 and GM2. The incubations were terminated by cooling the tubes in an ice bath, adding 400 μl of tetrahydrofuran and careful vortexing. The mixtures were then centrifuged at 10,000 × *g* for 5 min, and 5 μl of the resulting supernatants were subjected to HPTLC on silica gel plates with chloroform, methanol, 0.2% aqueous $CaCl_2$ (50:42:11, v/v/v) as a solvent system to separate the reaction products from the substrates. The separated glycolipids were quantified by radiochromatoscanning (Beta Imager 2000, equipped with the software interface Beta Vision, Dell Optiplex Systems, Biospace Mesures, Paris, France), and the enzyme activity was calculated as described (38).

Monomeric Dispersions of Gangliosides as Substrates—The assay of sialidase activity on monomeric dispersions of ^{3}H-labeled gangliosides (GM1 in most experiments; GD1a and GM2 in some experiments) was carried out as follows. The incubation mixtures (100 μl) set up in Eppendorf tubes and containing monomeric dispersions of ganglioside (concentration from 1.0×10^{-9} to 2×10^{-8} M) and 0.2 μg (at pH 5.6) or 1.5 μg (pH 7.0) of recombinant HsNEU2 were placed in an incubator shaker and incubated at 37 °C for the established time (usually 10 min). The enzyme reaction was stopped by immersing the tubes in an acetone/solid CO_2 bath and immediately freeze-dried. When different microbial sialidases were employed, the same amount of enzyme (0.2 μg) was incubated in the presence of 4×10^{-9} M ^{3}H-labeled GM1 for a proper period of time at the optimal pH described for the single bacterial sialidase.

The identification and quantification of the product of GM1 enzymatic hydrolysis were carried out by the already described HPTLC and column chromatography procedures. In the case of the HPTLC method, the freeze-dried residues were dissolved in 50 μl of chloroform/methanol (2:1, v/v), and the product of GM1 enzymatic hydrolysis was separated by HPTLC, quantified by radiochromatoscanning, and used to determine the total enzyme activity. The identification of the reaction product was performed by co-chromatography with standard ^{3}H-labeled GA1. In the case of the chromatographic method, the freeze-dried residues were dissolved with 100 μl of distilled methanol and quantitatively transferred upon DEAE-Sephadex A-25 columns (200 μl of swol-

len resin embedded in a 0.5 × 1.0-cm column) equilibrated in the same solvent. Separation of GA1 and GM1 was obtained using sequentially distilled methanol and 0.2 M sodium acetate in distilled methanol according to Iwamori and Nagai (40). The fractions were concentrated, and some aliquots were utilized to determine bound radioactivity in a liquid scintillation counter, and other aliquots were submitted to HPTLC in order to verify the efficacy of separation. The distribution of sialidase activity between the aqueous solution and the tube wall was established by separating the two fractions as described above and measuring formed radiolabeled GA1 by the HPTLC method.

Other Chemical Methods

Protein content was determined by the Coomassie Brilliant Blue method (41). Ganglioside-bound NeuAc was determined by the method of Svennerholm (22).

RESULTS

Expression and Purification of HsNEU2 in E. coli Cells—In order to facilitate the characterization of this human recombinant enzyme, we expressed it in *E. coli*, using the pGEX-2T vector (15) that provides HsNEU2 expression as a fusion protein with GST. HsNEU2 expression was easily detected on crude *E. coli* extracts, due to the lack of endogenous sialidase activity in these cells and the full conservation of catalytic power by the protein chimera GST-HsNEU2, which was also shown to exhibit a high degree of thermal stability. The fusion protein was purified from a crude bacterial lysate by affinity chromatography with GSH-Sepharose 4B. Cleavage of HsNEU2 from GST was achieved directly on the column using thrombin, whose recognition sequence is located immediately upstream of the ATG of the sialidase protein. Released HsNEU2 was directly eluted from the column with PBS and further purified by FPLC using Mono Q and Mono S ion exchange columns (Fig. 1). As shown in Fig. 2, the purified protein appeared as a single band on SDS-PAGE, with an apparent molecular mass of about 42 kDa, and exhibited a single band at pH 6.8 upon isoelectrofocusing, indicating a high degree of purity. The average recovery of recombinant HsNEU2 sialidase was about 0.9 mg protein starting from 1 liter of *E. coli* culture.

HsNEU2 Basic Biochemical Characterization

pH Optimum, Stability, and General Characteristics—The experiments concerning this point were carried out using MU-NeuAc as substrate. The purified recombinant HsNEU2 had a pH optimum 5.6, with appreciable activities in the pH range 5.0–7.2 (Fig. 3*A*) and was unstable upon freezing at −20 °C (50% loss after one cycle of freezing-thawing) and storage at 4 °C (50% loss after 24 h). Notably, the enzyme could be stored in PBS at −80 °C in small aliquots and thawed just before use without any significant loss of activity (assessed after 1 year). All measurements were carried out in the presence of bovine serum albumin (0.2 mg/ml), to overcome any possible enzyme inhibition exerted by liberated sialic acid. At the optimal pH, the enzyme reaction occurred linearly with incubation time up to 30 min and with the amount of protein up to 0.08 μg (Fig. 3, *B* and *C*). Na^+ and K^+ displayed slight activatory effects up to 10^{-5} M, Cu^{2+} showed a strong inhibition over 10^{-5} M, and Ca^{2+} showed no effect up to 10^{-3} M; all tested ions started inhibiting in a more or less pronounced way over 10^{-3} M (Fig. 3*D*). The enzyme kinetics obeyed regular Michaelis-Menten relationships (see Fig. 4*A*). At the optimal pH, the K_m and apparent V_{max} for MU-NeuAc were 0.44 mM and 47.6 IU/mg protein, respectively (Table I). On this basis, routine determinations were carried out using 0.01–0.02 μg of enzyme protein, 500 μM MU-NeuAc, and a 10-min incubation. *Ad hoc* experiments showed that the optimal pH (5.6) remained unchanged for all of the other substrates tested. In the case of substrates like colominic acid, glycoproteins, and oligosaccharides, where the enzyme activity detected was low or null, incubations were carried out with enzyme quantity up to 5 μg and incubation times up to 90 min.

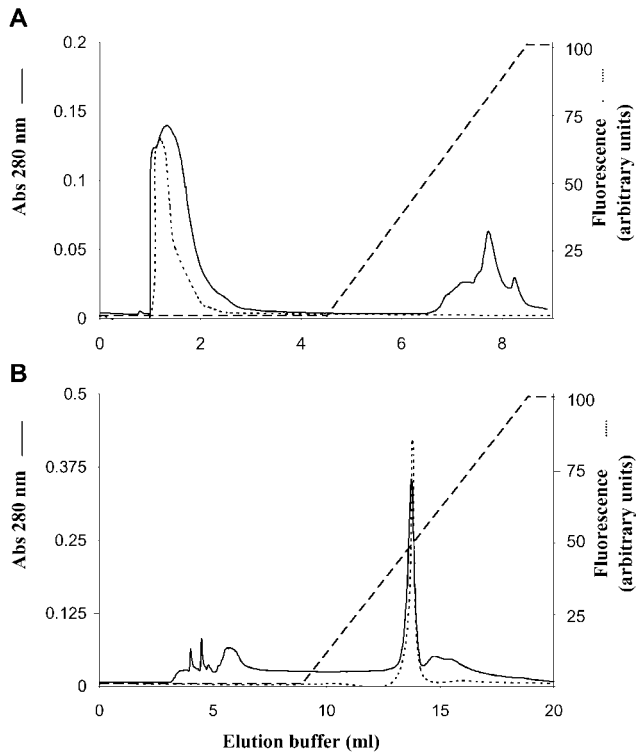

FIG. 1. **Elution profile of recombinant HsNEU2 on Mono Q and Mono S columns.** *A*, 1 mg (as protein) of the HsNEU2 preparation, obtained after GSH-Sepharose column chromatography and trombin splitting of GST, was submitted to FPLC chromatography on a Mono Q HR 5/5 column. *B*, the eluted fractions from the previous column carrying the enzyme, after dialysis and concentration under vacuum, were applied to a MONO S HR 5/5 column. The assay of sialidase was performed by the fluorimetric assay (substrate MU-NeuAc), and protein was determined by measuring absorbance at 280 nm. *Hatched line*, elution with 50 mM MES buffer, pH 6.7, carrying a linear NaCl gradient from 0 (after 9 ml of elution) to 1 M (after 19 ml of elution). For details, see "Experimental Procedures."

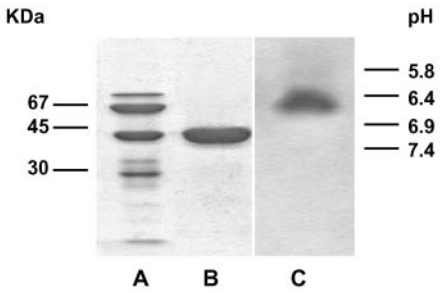

FIG. 2. **Analysis of purified HsNEU2, after Mono S HR 5/5 column chromatography.** *A*, SDS-PAGE of the product obtained after GSH-Sepharose column chromatography and trombin splitting of GST. *B*, SDS-PAGE of the product after MONO S HR5/5 FPLC. *C*, isoelectrofocusing of the product after MONO S HR5/5 FPLC. For details, see "Experimental Procedures."

Substrate Specificity—Table I shows the different sialyl-glycoconjugates tested as substrates for recombinant HsNEU2 and the corresponding K_m and apparent V_{max} values, measured after incubation at the optimal pH (5.6) and at pH 7.0. HsNEU2 acted on α2→3 sialyllactose but not on the α2→6 isomer nor on the α2→3 sialosyl linkage of the GM1 oligosaccharide, even using long incubation times (90 min) and high enzyme concentrations (5 μg). The activity of HsNEU2 on sialoglycoproteins was generally low, with the exception of fetuin; the apparent V_{max} at pH 5.6 decreased from a maximum of 12.7

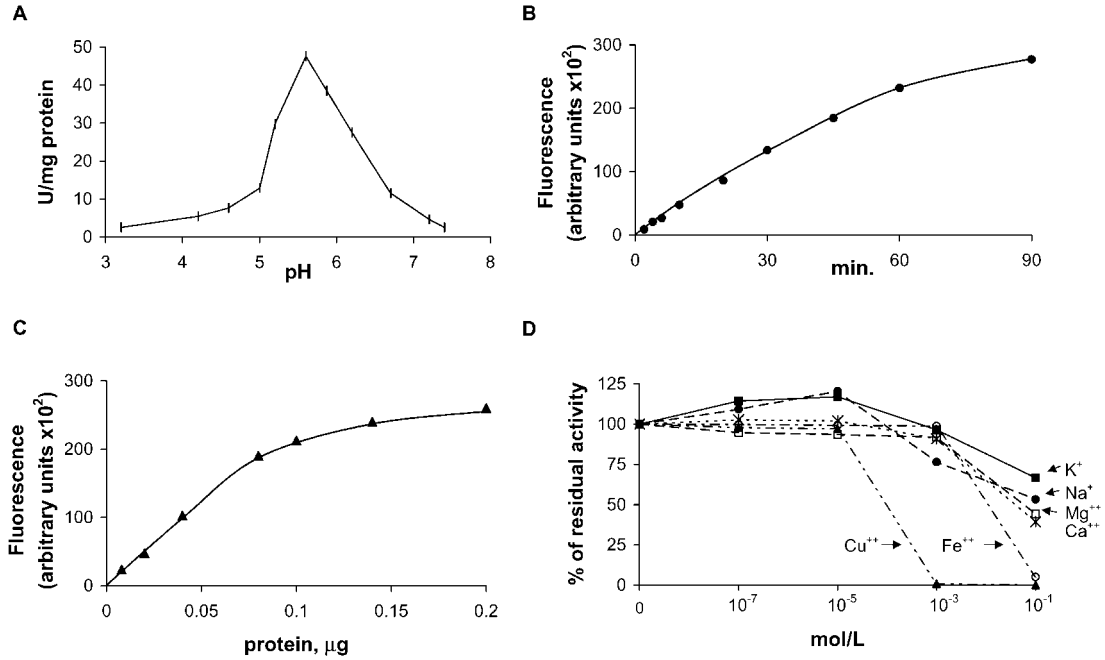

FIG. 3. **Basic properties of purified HsNEU2 ascertained using MU-NeuAc as substrate.** *A*, effect of pH. Assay conditions were as follows: 0.02 μg of enzyme; 0.5 mM substrate; incubation time, 10 min. *B*, effect of incubation time. Assay conditions were as follows: 0.02 μg of enzyme; 0.5 mM substrate; pH 5.6; increasing incubation time. *C*, relationship between enzyme activity and enzyme content. Assay conditions were as follows: 0.5 mM substrate; pH 5.6; increasing amounts of enzyme; incubation time, 10 min. *D*, effect of Cu^{2+}, Na^{+}, K^{+}, Ca^{2+}, Mg^{2+}, and Fe^{2+}. Assay conditions were as follows: 0.02 μg of enzyme; 0.5 mM substrate; pH 5.6; incubation time, 10 min; ions at different concentrations. For further details, see "Experimental Procedures." The data shown are the average of three experiments carried out in triplicate.

IU/mg protein (fetuin) to a minimum of 0.7 IU/mg protein (α_1-acid glycoprotein). The affinity for these substrates could not be measured accurately due to the paucity of the enzyme action. Since these glycoproteins contain $\alpha2\rightarrow3$ (fetuin), mixed $\alpha2\rightarrow3$ and $\alpha2\rightarrow6$ (α_1-acid glycoprotein), and $\alpha2\rightarrow6$ (transferrin) sialosyl linkages (to galactose), the detected activity confirmed the ability of HsNEU2 to hydrolyze $\alpha2\rightarrow3$ linked sialosyl residues in glycoproteins. However, the enzyme was also capable of hydrolyzing the $\alpha2\rightarrow6$ sialosyl bond of transferrin, differently from the $\alpha2\rightarrow6$ sialosyl linkage present in $\alpha2\rightarrow6$ sialyllactose. The enzyme did not seemingly discriminate between *N*- and *O*-glycosylated glycoproteins. Moreover, HsNEU2 did not cleave at all the $\alpha2\rightarrow8$ sialosyl linkage present in colominic acid. HsNEU2 removed with high efficiency sialic acid residues from gangliosides GD1a, GT1b, $\alpha2\rightarrow3$SPG, and GD1b (producing GM1, GD1b, asialo-SPG, and GM1, respectively). At the used concentrations (10^{-4} to 10^{-5} M), all of these gangliosides were present as micelles. Instead, using the same concentrations, the enzyme action on GM3 (vesicles) was extremely poor and completely null on GM1 and GM2, which also occur as micelles (see Table I).

The *V*/[S] curves (see examples in Fig. 4) obeyed typical Michaelis-Menten kinetics. The calculated K_m at the optimal pH had similar values for GD1b and GT1b (0.59 and 0.62×10^{-3} M), $\alpha2\rightarrow3$SPG (0.43×10^{-3} M), and GM3 (0.43×10^{-3} M) but a higher value for GD1a (0.91×10^{-3} M). The apparent V_{max} value, always at the optimal pH, was the highest for GD1a (250 IU/mg protein), followed by GT1b and $\alpha2\rightarrow3$SPG, and the lowest for GM3 and GD1b (7.8 and 7.5 IU/mg protein, respectively). This indicates that the enzyme prefers the $\alpha2\rightarrow3$-linked sialic acid residues at the terminus of the ganglioside oligosaccharide chain, although it is also able to hydrolyse the $\alpha2\rightarrow8$-linked sialic acid residues present in ganglioside GD1b. The apparent V_{max} of HsNEU2 on pure GM3 (which features a vesicular type of aggregation) is low anyway (7.8 mg protein), despite the presence of the $\alpha2\rightarrow3$ sialosyl linkage.

As shown in Table I, HsNEU2 expressed appreciable activity at pH 7.0 with all tested substrates on which the enzyme was capable of working. The ratio between the activity (V_{max}) at the optimal pH and pH 7.0, in the absence of detergents, ranged from a minimum of 1.55 with α_1-acid glycoprotein to a maximum of 24 with GD1a. With all substrates, the K_m values were practically identical at pH 5.6 and 7.0.

Effect of Detergents—The effect of neutral (Triton X-100 and octyl glucoside) and ionic (sodium deoxycholate and CHAPS) detergents on HsNEU2 activity was studied. None of these detergents (up to 0.5% concentration) influenced the enzyme activity on MU-NeuAc, sialyl-oligosaccharides, colominic acid, and all the tested sialoglycoproteins. Conversely, remarkable activatory effects were observed with gangliosidic substrates. The highest activation (1.5–2.5-fold, depending on the ganglioside) was observed with Triton X-100 at the optimal 0.02% (w/v) concentration, followed by octyl glucoside (1.3–2.2-fold at 0.08% w/v) and sodium deoxycholate (1.1–1.8-fold at 0.03% w/v). The only exception was GD1b, where all detergents caused a small decrease of the apparent V_{max}.

The activity of HsNEU2 toward mixed micelles of ganglioside/Triton X-100 followed again regular Michaelis-Menten kinetics (see, for example, the case of GM3 (Fig. 4)). In any case, the most striking effect of the detergent was detectable just on ganglioside GM3, where the presence of Triton X-100 caused a more than 8-fold increase of apparent V_{max}. Moreover, the presence of detergent increased the affinity, as compared with homogeneous micelles. This increase was highest, about 6-fold, with GD1a. Notably, none of the used detergents, in a wide range of concentrations, enabled HsNEU2 to work on micellar GM1 and GM2.

The effect of Triton X-100 was present also at pH 7.0. At this pH, the increase of enzyme activity due to Triton X-100 was markedly higher than at the optimal pH. In the case of GM3, the activity increased 8.6-fold at pH 5.6 and 13.7-fold at pH 7.0, and in that of GD1b, the detergent caused a substantial in-

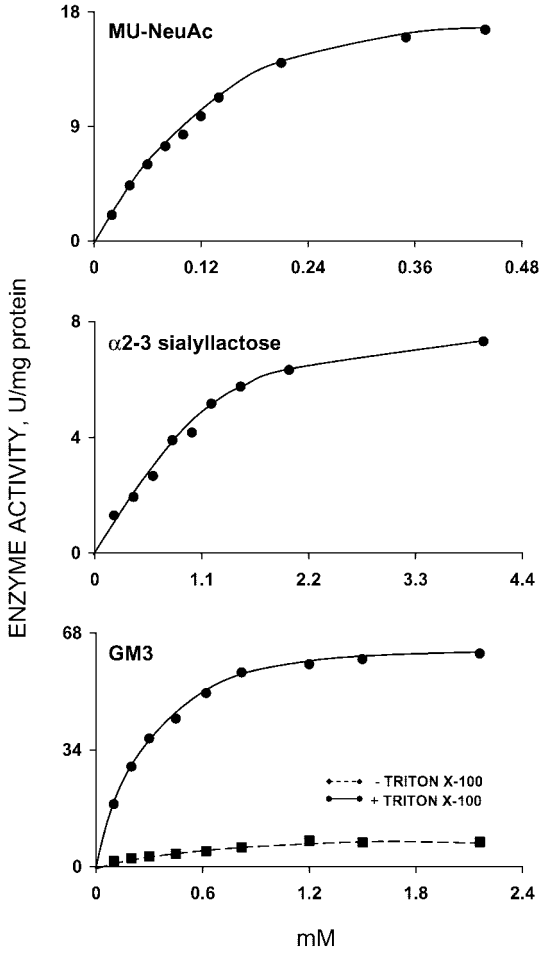

FIG. 4. **Kinetics of HsNEU2 on various substrates (MU-NeuAc, α2→3 sialyllactose, and GM3).** Assay conditions were as follows: 0.02 μg of enzyme; pH optimum, 5.6; incubation time, 10 min; Triton X-100 (when present), 0.4 mM. The enzyme activity was measured by the fluorimetric method with MU-NeuAc, by the colorimetric method with α2→3 sialyllactose, and by the radiometric method with GM3. The data shown are the average of two experiments carried out in triplicate.

crease (2.7-fold) against an almost 30% decrease at pH 5.6. Also, in the presence of detergent, the K_m values did not undergo significant changes from pH 5.6 to 7.0.

HsNEU2 Activity toward Monomeric Dispersions of GD1a and GM1 Gangliosides

After having established the ability of recombinant HsNEU2 to hydrolyze micellar gangliosides, particularly GD1a (that can be radiolabeled with high specific radioactivity), we performed experiments aimed at ascertaining the possible action of the enzyme on the monomeric form of GD1a that is at concentrations below 10^{-8} M. For this purpose, we used the radiochemical assay method, the only method possessing adequate sensitivity. As shown in Fig. 5, GM1 was formed as the principal reaction product in a time-dependent manner, indicating the ability of HsNEU2 to hydrolyze monomeric GD1a. However, the same experiments showed the formation of an additional radioactive compound with a slightly higher R_F than GM1 in our HPTLC separation system. This compound exhibited the same Rf of standard asialo-GM1 (GA1) (Fig. 5), and its identification as GA1 was accomplished using both HPTLC co-migration experiments and ion exchange chromatography on DEAE-Sephadex A25, which differentiates the sialylated and not sialylated glycolipid. Notably, also at pH 7.0 (Fig. 5), HsNEU2 was able to catalyze the hydrolysis of GD1a into GM1 and of formed GM1 into GA1. Subsequent studies performed at nanomolar concentrations of [^{3}H]GM1 at the optimal pH (see Fig. 6) showed that the formation of GA1 from monomeric GM1 was time- and enzyme concentration-dependent. At this point, a consideration had to be made. Since, at the used concentrations, the ganglioside is present in the monomeric form and since monomeric gangliosides are known to adhere to the wall of the test tube, the distribution of the GM1 substrate as well as of the GA1 reaction product between the aqueous solution and the tube wall had to be investigated. The results are shown in Table II. Before incubation with HsNEU2, GM1-bound radioactivity was almost completely linked to the tube wall, an almost negligible portion being measurable in the water solution. After incubation, 97.5% of total radioactivity was again linked to the tube wall, and 1.8% was in the water solution. The distribution of GM1 and formed GA1 into the two fractions was parallel, and the sialidase activity was measurable only on the tube wall, with a recovery of 95.7%, indicating that the enzyme acted on tube wall-linked GM1, and the formed GA1 remained linked to the tube wall. Surprisingly, despite the very low substrate concentration, the *V*/[S] curve seemingly showed a hyperbolic course, and the apparent V_{max} and K_m values could be determined with an acceptable degree of approximation: about 10×10^{-9} M and 25 μIU/mg protein, respectively (Fig. 7*A*). Moreover, pilot experiments showed that HsNEU2 was able to affect [*Sph*-^{3}H]GM2 at 2–5 nM concentrations with formation of a compound migrating a little faster than GM2 and lacking the acidic charge, presumably asialo-GM2.

Hydrolysis of Monomerically Dispersed GM1 in Triton X-100 Micelles

The capability of Triton X-100 (CMC about 10^{-4} M) to promote the passage of monomerically dispersed GM1 from the reaction tube walls to the aqueous solution was ascertained. As shown in Table III, using a fixed amount of radioactive GM1 (1 nM) and increasing amounts of Triton X-100 (from 3.9 to 396 μM), a progressive passage of the ganglioside into the aqueous phase was observed. Actually, using 396 μM detergent (*i.e.* about 0.4 mM), ~97% of the total radiolabeled GM1 was recovered in the aqueous solution, and only 1.7% bound to the tube wall. On the other hand, maintaining constant detergent concentrations (0.4 mM) and increasing the quantity of radioactive GM1 from 1.0 to 40.2 nM (Table III, bottom), almost all of the ganglioside (about 97%) remained in the aqueous phase. Finally, as shown in Table IV, the sialidase activity on GM1, which was almost completely (95.7%) linked to the tube wall in the absence of Triton X-100, moved to the aqueous solution with increasing amounts of added detergent reaching 97.5% at 0.4 mM detergent concentration. Using monomeric dispersions of GM1 on the surface of Triton X-100 micelles (GM1/Triton X-100 molar ratio, 1:2000) the *V*/[S] relationships obeyed typical Michaelis-Menten kinetics, with K_m and V_{max} values of 15 nM and 72 μIU/mg protein, respectively (see Fig. 7*B*). The data shown in Fig. 7, *A* and *B*, show that the kinetics of HsNEU2 on monomerically dispersed GM1, on the tube wall or on the surface of Triton X-100 micelles, had the same trends at pH 5.6 and 7.0. The apparent V_{max} value was obviously lower at pH 7.0 than 5.6 (25 *versus* 72 IU/mg protein), whereas the K_m values were identical.

Action of Various Microbial Sialidases on GM1 Monomeric Dispersions: Comparison with HsNEU2—As shown in Fig. 8, the activity on monomeric dispersions of [^{3}H]GM1 also was displayed by some microbial sialidases. In fact, using the same incubation conditions as for the HsNEU2 assay (4 nM [^{3}H]GM1, 0.2 μg of enzyme, 10-min incubation at 37 °C, pH 5.6) and the

TABLE I

Kinetics parameters of HsNEU2 action on different substrates

Assay conditions were as follows: sialidase assay mixture, 50 mM sodium citrate-phosphate buffer; substrate at saturating concentrations; 0.02 μg of enzyme at pH 5.6 (established to be the optimal pH) and 0.15 μg at pH 7.0; incubation time at 37 °C up to 30 min depending on the substrate and pH (for more details, see "Experimental Procedures"). In the case of GM1, GM2, colominic acid, α2→6 sialyllactose, and GM1 oligosaccharide, different substrate concentrations were used as well as enzyme amounts up to 5 μg and incubation times up to 90 min. Sialidase activity on GM1, GM2, GM3, and GD1a was assayed by the radiochemical assay; activity on GD1b, GT1b, and α2→3SPG was assayed by the colorimetric assay; and that on MU-NEUAc was assayed by the fluorometric assay.

Substrate	Triton X-100 (0.02%, w/v)	V_{max}		K_m (10^{-3} M)[a]
		pH 5.6	pH 7.0	
		IU/mg of protein		
Gangliosides				
GM3	−	7.80	2.23	0.40
	+	67.00	30.70	0.24
GD1a	−	250.00	10.41	0.91
	+	322.00	22.50	0.14
GD1b	−	7.50	0.80	0.59
	+	5.45	2.20	0.51
GT1b	−	133.00	17.50	0.62
	+	190.00	27.20	0.38
α2→3SPG	−	105.00	12.22	0.43
	+	253.00	29.12	0.28
GM1	−	ND[b]	ND	ND
	+	ND	ND	ND
GM2	−	ND	ND	ND
	+	ND	ND	ND
Glycoproteins				
Fetuin	No effect[c]	12.70	3.40	Not established[d]
Transferrin	No effect	0.75	0.40	Not established
α_1-acid glycoprotein	No effect	0.70	0.45	Not established
Mucin[e]	No effect	1.82	0.17	
Colominic acid	No effect	ND	ND	ND
Oligosaccharides				
α2→3 sialyllactose	No effect	10.00	2.12	0.31
α2→6 sialyllactose	No effect	ND	ND	ND
GM1-oligosaccharide	No effect	ND	ND	ND
Artificial substrate				
MU-NeuAc	No effect	47.60	14.17	0.44

[a] No variations were detectable between acidic and neutral pH values.
[b] ND, not detectable.
[c] No effect, no effect by Triton X-100 was observed.
[d] Not established, it could not be established in an accurate way.
[e] From bovine submaxillary glands.

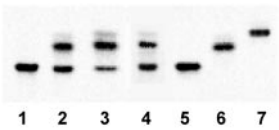

FIG. 5. **HsNEU2 activity on monomeric dispersions of [*Sph*-^{3}H]GD1a.** Enzyme assay conditions, at 37 °C, were as follows: 2 nM [*Sph*-^{3}H]GD1a; 0.2 μg of enzyme and 10–30-min incubation at pH 5.6; 1.2 μg of enzyme and 30-min incubation at pH 7.0. *Lane 1*, blank mixture (without enzyme); *lanes 2* and *3*, mixture incubated at pH 5.6 (10 and 30 min, respectively); *lane 4*, mixture incubated at pH 7.0; *lane 5*, standard [*Sph*-^{3}H]GD1a; *lane 6*, standard [*Sph*-^{3}H]GM1; *lane 7*, standard [*Sph*-^{3}H]GA1.

pH reported to be optimal for the individual bacterial sialidases (5.5 for *V. cholerae* sialidase; 5.6 for the remaining ones), formation of radiolabeled GA1 was observed in the case of *A. ureafaciens* and *C. perfringens* sialidases. The activities of these enzymes corresponded to about 48 and 3.8% of that displayed by HsNEU2, respectively. No activity at all on monomeric GM1 was detectable with *S. typhimurium* and *V. cholerae* sialidases.

DISCUSSION

The mammalian sialidases so far cloned (9, 10) show remarkably similar amino acid sequences and a highly conserved active site. However, they have different biological origin and subcellular localization, display different biological implications, and exhibit different substrate specificities. The molecular features responsible for these behavioral differences are unknown. The first aim of the present investigation was to better understand the sialidase specificity toward gangliosides, natural substrates of amphiphilic nature, in comparison with companion sialoglycoproteins and sialooligosaccharides. The particular attention to gangliosides had the following grounds: (i) gangliosides show characteristic aggregation properties in aqueous media, since they undergo monomer to micellar transition at very low critical micellar concentrations, 10^{-8} to 10^{-7} M, with the exception of GM3, which adopts a vesicular form of aggregation; (ii) they occur in membranes either in a packed organization (lipid rafts) (20, 21), resembling a micellar organization or in molecular dispersion as monomers; and (iii) they are involved in important events of social cell behavior, like cell-cell interactions, receptor regulation, and transmembrane signaling (42, 43), where their molecular and supramolecular features may play a crucial role. For the first time, the recombinant human cytosolic sialidase HsNEU2 was employed as a model enzyme.

HsNEU2 was expressed in *E. coli* cells as a protein chimera with GST with the advantage of grossly purifying it by column affinity chromatography on GSH-Sepharose, using GST as the binding portion, and of having an active and stable sialidase. After two additional chromatographic passages on FPLC, with concomitant detachment of GST by trombin treatment, the enzyme was homogeneous although thermolabile (unless stored at −80 °C). The pH optimum was confirmed to be 5.6 on all of the used substrates, as already reported for HsNEU2-transfected cells (15), the *V*/*E* and *V*/*t* relationships had the expected features, and the *V*/[S] relationships obeyed Michaelis-Menten kinetics. The detailed study on the enzyme sub-

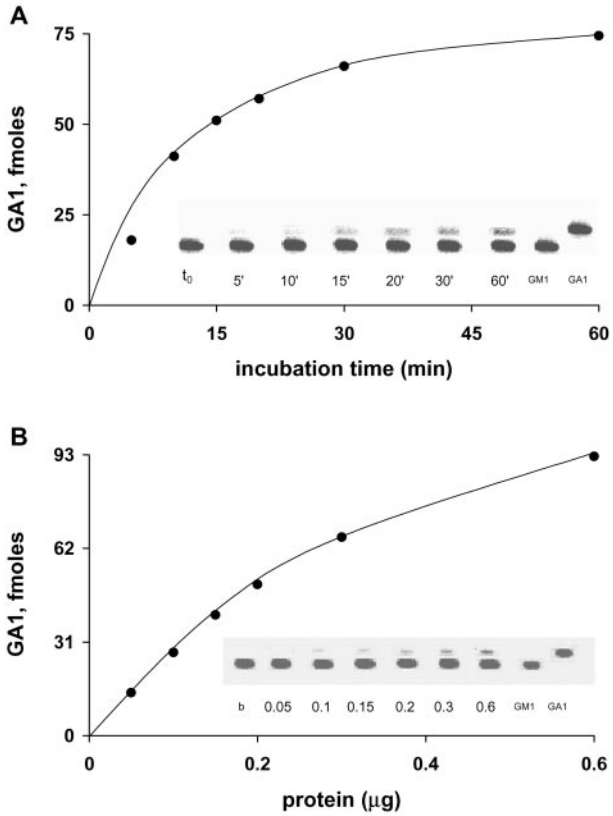

FIG. 6. **Effect of incubation time and enzyme content on HsNEU2 action on monomeric dispersions of GM1.** *A*, effect of incubation time. Assay conditions were as follows: 0.2 μg of enzyme; 1 nM [*Sph*-^{3}H]GM1; pH 5.6, 37 °C. *B*, relationship between enzyme activity and enzyme content. Assay conditions were as follows: 1 nM [*Sph*-^{3}H]GM1; pH 5.6, 37 °C; incubation time, 20 min. The data shown are the average of two experiments carried out in triplicate.

strate specificity provided some novel pieces of information. The first message concerned the linkage specificity, namely the sialosyl linkages $\alpha2\rightarrow3$ and $\alpha2\rightarrow6$ to galactose and $\alpha2\rightarrow8$ to a further sialic acid residue. The highest activity and affinity were observed on the $\alpha2\rightarrow3$ linkage-containing glycoconjugates, followed by $\alpha2\rightarrow8$ and $\alpha2\rightarrow6$ linkages, although with some relevant exceptions. In fact, the $\alpha2\rightarrow3$ linkage present in the GM1 oligosaccharide and in micellar GM1 and GM2 was resistant to the enzyme action as well as the $\alpha2\rightarrow8$ linkage present in colominic acid and the $\alpha2\rightarrow6$ linkage present in $\alpha2\rightarrow6$ sialyllactose. This means that a portion of the substrate molecule other than the sialosyl residue plays an important role in catalysis, suggesting the presence in HsNEU2 of additional sites (peptide domains or single amino acid residues sterically vicinal to the active site) involved in substrate recognition. The second message concerned the effect on the enzyme action of neutral and ionic detergents, the most active being Triton X-100. The detergent effect was observed only on ganglioside substrates, indicating its action at the substrate, not enzyme, level. At the optimal pH, and using millimolar ganglioside concentrations, largely above the CMC, hence in their micellar form, the detergent effect consisted in an increase of apparent V_{max} (1.7-fold in the case of GD1a, where the activatory effect was the highest) and decrease of K_m (the highest decrease, about 6.5-fold, again on GD1a). GD1b was the only ganglioside where the effect of detergent was almost null. Instead, GM3 showed the highest change in apparent V_{max} (8.5-fold rise), in the presence of detergent very likely due to its transition from the vesicular form of aggregation to the (mixed) micellar one with Triton X-100. Remarkably, the micelles of GM1 and GM2 were completely resistant to HsNEU2 action, regardless of the presence or absence of detergents. As a whole, these data are consistent with the notion that HsNEU2 is capable of recognizing not only the type of sialosyl linkage and the aglycone part of the molecule, but also the supramolecular organization (micelles and vesicles) of sialoglycoconjugates of an amphiphilic nature.

The third novel aspect of HsNEU2 is its peculiar ability to remove sialic acid from gangliosides in the concentration range 10^{-9} to 10^{-8} M, which is their monomeric form. This was observed with GD1a but also, and particularly, with GM1, which was studied in detail, and GM2. The very important difference was that in the case of GD1a, the enzyme works also on the micellar aggregation, whereas with GM1 and GM2 it absolutely does not. It should be recalled that at very low concentrations, the ganglioside substrate is not free in the aqueous reaction mixture but bound to the test tube wall. Despite this, recombinant HsNEU2 affects GM1 with the expected dependence on time and enzyme amount and with a *V*/[S] curve obeying Michaelis-Menten kinetics. The apparent V_{max} was 25 μIU/mg protein, and the K_m was as low as 10 nM, which is quite surprising for an enzyme. Moreover, the enzyme activity on monomeric dispersions of GM1 occurs also in the presence of Triton X-100. In this case, detergent addition leads to complete "solubilization" (detachment from the tube wall) of the monomeric GM1, probably because ganglioside is incorporated into the surface of Triton X-100 micelles, which are soluble. It seems reasonable to suggest that recombinant HsNEU2 is able to recognize ganglioside molecules dispersed on surfaces. Using Triton X-100/GM1 micelles with a molar ratio of 2000:1, which is a very dispersed form of GM1, the apparent K_m was still very low (15 nM) and V_{max} was raised to 72 μIU/mg protein.

A further aim of the investigation was to quantify the capability of HsNEU2 to work at a neutral pH. It was surprising to observe that the enzyme displayed, on all tested substrates and in the absence of Triton X-100 a substantial activity at pH 7.0, ranging (with reference with that at pH 5.6) from a minimum of 4% with GD1a to a maximum of 64% with α_1-acid glycoprotein. Using ganglioside substrates, in the presence of Triton X-100, the activatory effect of detergent tended to be relatively more effective at pH 7.0 than 5.6. In fact, the percentage of activity in reference to that at the optimal pH, rose from 4 to 7% with GD1a, from 28 to 45% with GM3, and from 10.7 to 40.3% with GD1b. The K_m values were in all cases identical at two pH values, indicating that the affinity of the enzyme was not affected by pH within the range used. Notably, also the ability of the enzyme to recognize monomeric dispersions of ganglioside substrates and to affect monomeric dispersions but not micellar aggregations of GM1 was expressed also at 7.0, with constant K_m and a 30–40% decrease of apparent V_{max}.

The accessibility or inaccessibility of the sialic acid of GM1 (and GM2) to sialidase action is an old and controversial issue. The lack of accessibility exhibited by some bacterial (*V. cholerae*), viral (influenza virus), and mammalian sialidases (1) was assumed to derive from a steric hindrance by the vicinal *N*-acetylhexosamine residue preventing the enzyme from properly interacting with the substrate (44). In other words, the constraint was viewed as residing at the substrate level. However, other sialidases of bacterial and mammalian origin resulted in the ability to promote sialic acid removal from GM1 and GM2 (45–49), pointing to structural features at the enzyme level. Notably, Wang *et al.* (50) showed that proper point mutations on recombinant rat plasma membrane-bound sialidase markedly enhanced the ability of the enzyme to hydrolyze GM2. An important contribution to clarify the issue comes from

TABLE II

Action of HsNEU2 on monomeric dispersions of ^{3}H-labeled GM1: Evidence for the enzyme activity on tube wall-bound gangliosides

Assay conditions were as follows: HsNEU2, 0.4 μg; 1 nM [*Sph*-^{3}H]GM1; 50 mM citrate/phosphate buffer, pH 5.6; 30 min at 37 °C. Separation of GM1 (and GA1, after incubation) between the tube wall-bound form and the form present in the aqueous solution was performed as described under "Experimental Procedures." The data are the means ± S.D. of three experiments carried out in triplicate. The same radioactivity distribution pattern was observed at pH 7.0, using 1.2 μg of enzyme.

	Before incubation		After incubation			
	Total radioactivity	Percentage	Total radioactivity	Percentage	Sialidase activity	Percentage
	dpm	*%*	*dpm*	*%*	*μIU/mg of protein*	*%*
Aqueous solution	53 ± 3	1.7	58.4 ± 4	1.83	ND[a]	
Test tube wall	3120 ± 240	98.1	3100 ± 280	97.5	1.31 ± 0.09	95.7
Total	3180 ± 159	100	3180 ± 159	100	1.37 ± 0.07	100
Recovery (%)		99.8		99.33		95.7

[a] Not detectable.

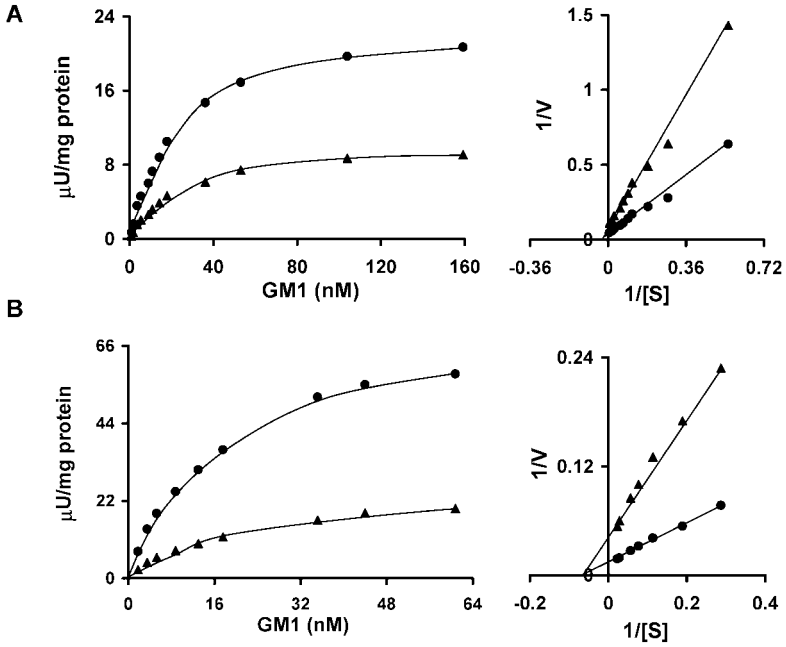

FIG. 7. **Kinetics of HsNEU2 on monomeric dispersions of GM1 in the absence of Triton X-100 (dispersion on the tube wall) (*A*) or presence of Triton X-100 (dispersion on the detergent micelle) (*B*).** Enzyme assay conditions were as follows: 0.4 mM Triton X-100, when present; [*Sph*-^{3}H]GM1 at different concentrations; 0.2 μg of enzyme and 10 min incubation at pH 5.6; 1.2 μg enzyme and 30 min incubation at pH 7.0. The data shown are the average of two experiments carried out in triplicate. ●, pH 5.6; ▲, pH 7.0.

the reported resolution of the three-dimensional structure of human GM2 activator protein (GM2-AP) (51). This protein is a cofactor in the intralysosomal degradation of gangliosides, and its genetically determined defect leads to a variant of Tay-Sachs disease known as AB GM2 gangliosidosis (52). The GM2-AP crystal structure reveals a β-cup-shaped folding, with a central hydrophobic cavity, which is suitable for binding the ceramide lipid tail of gangliosides, namely GM2. The activator protein probably extracts single ganglioside molecules from the membrane and confers on the head saccharide moiety a new conformation that renders it accessible to the enzyme, in this case a β-*N*-acetylhexosaminidase. This model can be applied also to the GM1/GM2 sialidase interactions. Actually, in a recent study, mouse plasma membrane-associated sialidase (MmNEU3) was shown to remove sialic acid from GM1 and GM2 in the presence of GM2-AP but also, although with a much lower efficiency, in the absence of the activator (49). A possible explanation for all of these different behaviors is that the oligosaccharide portion of gangliosides, like GM1 and GM2, should have a particular conformation relative to the hydrophobic tail, in order to be recognized and hydrolyzed by sialidase. This conformation can simply be met by ganglioside molecules dispersed on a surface (as in the case of recombinant HsNEU2) or by ganglioside molecules inserted into the hydrophobic cavity of GM2-AP. For the case where sialidases seemingly act on GM1 and GM2 micelles, the hypothesis can be put forward that the right substrate conformation is obtained through a preliminary interaction of the substrate with a binding site of the enzyme itself acting somewhat like the GM2-AP. The availability of different recombinant sialidases, properly engineered by selected point mutations, might contribute to verify this line of interpretation.

The importance of structural differences among sialidases, especially with regard to substrate specificity and kinetics parameters, is also stressed by differences in the action of recombinant HsNEU2 and the other mammalian cytosolic sialidases purified from rat liver (53) and Chinese hamster ovary cells (54). For instance, the latter enzymes, differently from HsNEU2, have a pH optimum of 6.0, recognize as substrates α2→6 sialyllactose and colominic acid, and display a higher activity on transferrin than fetuin and a relatively lower activity on (micellar) gangliosides. Instead, they share with HsNEU2 the inability to hydrolyze micellar GM1 and GM2. It is unknown whether these sialidases are able to affect gangliosides as monomeric dispersion. The reason for all of these differences may reside in structural differences or, in some cases, the different analytical procedures employed or seemingly because the issue (it is the case of ganglioside monomeric dispersions) was not addressed.

Finally, the possible link between the results provided by this study and the possible physiological significance of cytosolic sialidases in mammalian cells deserves comment. Transfection of rat B16 melanoma cells with rat NEU2 cDNA was reported (55) to suppress pulmonary metastases, and the met-

TABLE III

Triton X-100 mediates the passage of monomeric dispersions of GM1 from the test tube wall to the aqueous medium

Experimental conditions were as follows. Top, [*Sph*-^{3}H]GM1, 1 nM; Triton X-100, from 0 to 396 μM; 50 mM sodium citrate phosphate buffer, pH 5.6; final volume, 0.1 ml; 37 °C. Bottom, [*Sph*-^{3}H]GM1, from 1 to 40.2 nM; Triton X-100, 396 μM; 50 mM sodium citrate/phosphate buffer at pH 5.6; final volume, 0.1 ml; 37 °C. The mixtures were prepared, and the distribution of GM1 molecules between the Eppendorf test tube wall and the aqueous solution was determined as described under "Experimental Procedures." The data shown are the mean values ± S.D. of three experiments carried out in triplicate. No cold GM1 was used. Starting GM1-bound radioactivity was 3180 dpm. No significant difference in the distribution of radioactivity into the test tube wall and aqueous solution was observed at pH 7.0.

Triton X-100	GM1-bound radioactivity			
	Aqueous solution		Test tube wall	
	Radioactivity	Percentage	Radioactivity	Percentage
μM	*total dpm*	*%*	*total dpm*	*%*
0	53 ± 3	1.7	3120 ± 159	98.1
3.9	700 ± 45	22.0	2400 ± 122	75.5
33.0	1220 ± 72	38.4	1900 ± 101	59.7
99.0	1890 ± 98	59.4	1050 ± 55	33.0
198.0	2660 ± 130	83.6	480 ± 22	15.1
297.0	3010 ± 138	94.6	80 ± 5	2.5
396.0	3090 ± 141	91.2	50 ± 2	1.6

GM1		GM1-bound radioactivity			
		Aqueous solution		Test tube wall	
Concentration	Radioactivity	Radioactivity	Percentage	Radioactivity	Percentage
nM	*total dpm*	*total dpm*	*%*	*total dpm*	*%*
1.0	3180	3090 ± 141	97.2	50 ± 2	1.6
1.3	4134	4304 ± 122	97.4	58 ± 3	1.4
5.05	16,059	15,593 ± 855	97.1	321 ± 18	2.0
9.02	28,684	27,709 ± 1434	96.6	946 ± 51	3.3
19.7	62,646	60,641 ± 3217	96.8	1940 ± 142	3.1
40.2	127,836	123,617 ± 5870	96.7	3068 ± 153	2.4

TABLE IV

Action of HsNEU2 on monomeric dispersions of GM1: Effect of Triton X-100-mediated passage of GM1 monomers from the test tube wall to the aqueous solution

Experimental conditions were as follows: [*Sph*-^{3}H]GM1, 1 nM; Triton X-100, from 0 to 396 μM; 50 mM citrate/phosphate buffer, pH 5.6; HsNEU2, 0.4 μg; final volume, 0.1 ml; incubation time, 10 min at 37 °C; separation of formed [*Sph*-^{3}H]GM1 by HPTLC; radioactivity measurement by radiochromato scanning. For details, see "Experimental Procedures." The data shown are the mean values of two experiments carried out in triplicate. Starting total sialidase activity was 0.55 nIU. The same trend of enzyme activity distribution in the aqueous solution and test tube wall was observed at pH 7.0 (1.2 μg of enzyme).

Triton X-100	Sialidase activity			
	Aqueous solution		Test tube wall	
	Activity	Percentage	Activity	Percentage
nM	*total nIU*	*%*	*total nIU*	*%*
0	ND[a]		0.526	95.7
3.9	0.124	22.5	0.412	74.9
33.0	0.209	38.1	0.331	60.2
99.0	0.330	60.1	0.171	32.4
198.0	0.457	83.1	0.086	15.7
297.0	0.522	94.9	0.012	2.2
396.0	0.543	97.7	ND	

[a] ND, not detectable.

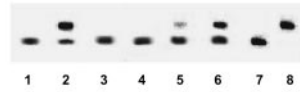

FIG. 8. **Action of different sialidases on monomeric dispersions of [*Sph*-^{3}H]GM1.** Enzyme assay conditions were as follows: 0.2 μg of enzyme; 1 nM [*Sph*-^{3}H]GM1; incubation time, 10 min; optimal pH, 5.5 for *V. cholerae* sialidase and 5.6 for the remainder ones; 37 °C. *Lane 1*, blank (without enzyme); *lane 2*, with HsNEU2; *lane 3*, with *S. typhimurium* sialidase; *lane 4*, with *V. cholerae* sialidase; *lane 5*, with *C. perfringens* sialidase; *lane 6*, with *A. ureafaciens* sialidase; *lane 7*, standard [*Sph*-^{3}H]GM1; *lane 8*, standard [*Sph*-^{3}H]GA1. When *S. typhimurium* and *V. cholerae* sialidase were employed, 1 μg of enzyme was added, and the incubation time was extended to 60 min.

astatic potential of sublines of mouse colon adenocarcinoma was inversely proportional to the level of endogenous NEU2 (56). On the other hand, transfection of human epidermoid carcinoma cells with hamster NEU2 matched with enhancement of epidermal growth factor receptor activity (57) and overexpression of rat NEU2 in rat myoblasts led to promotion of differentiation toward myotubules (58). Taken together, these data indicate that whenever an increase or appearance of NEU2 activity was observed, a decrease of GM3 and an increase (or appearance) of lactosylceramide, the product of GM3 desialosylation, were detected. Since both GM3 and lactosylceramide are involved in transmembrane signaling pathways (59), generally with opposite biological effects, a role of sialidase as a regulatory switch can be easily suggested. The fact that HsNEU2 displays activity also at a neutral pH removes, at least in part, the objection of what can be the physiological role of a cytosolic enzyme having an optimal pH in the acidic range. Instead, it can be suggested that a transient acidification of a microenvironment of the cytosol may constitute an efficient mechanism of *in vivo* enzyme regulation.

Assuming gangliosides as a main target for cytosolic sialidase and considering that the assumed intracellular localization of gangliosides is to the external surface of the plasma membrane or the luminal surface of Golgi membrane, the problem of substrate accessibility to the enzyme is pivotal. The possibility that under proper conditions the cytosolic enzyme can leave its residential compartment and reach ganglioside cannot be excluded. In fact, it was shown (60, 61) that in human cultured fibroblasts and Chinese hamster ovary cells, NEU2 could be released in the medium and reach surface-linked ganglioside. Moreover, small amounts of gangliosides are associated with intermediate filaments and microtubes (62) or are present in the cytosol in the form of protein complexes (63) and, as such, can be reached by the cytosolic sialidase. At this level, the ability of the enzyme to act on either monomerically dispersed or "clustered" gangliosides, although with quite different affinity and efficiency, might be critical for the dynamics

and function of lipid rafts. Of course, huge efforts must be made to properly approach and make clear these important issues.

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