Characterization of human placental neuraminidases

Stability, substrate specificity and molecular weight

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1. At least two components of neuraminidase can be distinguished on the basis of thermostability and sedimentability by using the artificial fluorogenic substrate 4-methylumbelliferyl *N*-acetyl- α -D-neuraminatate. 2. In crude homogenates, thermodenaturation at 25°C showed a biphasic curve corresponding to component A (half-life, 21 min) and B (half-life, 85 min). The two components were partially resolved by centrifugation, A being soluble and B sedimentable. Both had similar pH-activity curves (pH optimum, 4.4), K_m values (A, 0.10 mM; B, 0.06 mM) and molecular weight as determined by radiation inactivation (A, 67 000; B, 63 000). 3. The soluble A form was still aggregated or bound to membranous debris since almost all neuraminidase activity was eluted near or at the void volume of a Sephacryl S-300 column. 4. Both soluble and sedimentable fractions of placenta hydrolysed the G_{D1A} ganglioside and *N*-acetylneuraminyl-D-lactose linearly for 12 h but no fetuin hydrolysis was detected. 5. The neuraminidase activity with the artificial fluorogenic substrate was inhibited by *N*-acetylneuraminyl-D-lactose but not by the G_{D1A} ganglioside. These preliminary results suggest that there exist two closely related enzymes hydrolysing both the artificial substrate and *N*-acetylneuraminyl-D-lactose and a third one hydrolysing the G_{D1A} ganglioside exclusively.

Neuraminidase (acylneuraminyl hydrolase, EC 3.2.1.18) hydrolyses terminal sialic acid residues from glycoproteins, oligosaccharides and glycolipids. In rat liver, the enzyme has been localized in lysosomes (Aronson & de Duve, 1968; Horvat & Touster, 1968), Golgi apparatus (Kishore *et al.*, 1975), plasma membrane (Shengrun *et al.*, 1972) and cytosol (Tulsiani & Carubelli, 1970). Substrate specificity of the lysosomal and plasma-membrane neuraminidase is directed to both oligosaccharides and gangliosides, whereas the enzyme of Golgi apparatus and cytosol hydrolyses only the oligosaccharides. The cytosolic neuraminidase is soluble but other neuraminidases associated with intracellular organelles are membrane-bound, and difficult to obtain in a soluble and active form (Ohman *et al.*, 1970). However, neuraminidase was purified and partially characterized in pig brain (Venerando *et al.*, 1975), rat heart (Tallman & Brady, 1973), rabbit spermatozoal acrosomes (Srivastava & Abou-Issa, 1977), rabbit endometrium (Srivastava

& Farooqui, 1980), cultured skin fibroblasts (Shengrun *et al.*, 1973; Caimi *et al.*, 1979; Thomas *et al.*, 1979; Zeigler & Bach, 1981), human liver (Corfield *et al.*, 1981; Veh & Sander, 1981) and brain tissues (Carubelli *et al.*, 1962; Leibovitz & Gatt, 1968; Ohman *et al.*, 1970; Venerando *et al.*, 1975; Sandhoff & Pallman, 1978). The molecular weight of a human neuraminidase was reported in the leucocytes (203 000–240 000; Nguyen *et al.*, 1980).

Recent interest in human neuraminidase was stimulated by the discovery of several human genetic disorders associated with a deficiency of this enzyme. The sialidoses, or neuraminidase-deficiency syndromes, include a normosomatic group of patients [the cherry-red spot syndrome with or without myoclonus (Durand *et al.*, 1977; O'Brien, 1977)] and a dysmorphic group with juvenile onset [the Goldberg syndrome (Myers *et al.*, 1980)], the cherry-red spot myoclonus syndrome with dementia and associated β -galactosidase deficiency (Wenger *et al.*, 1978) and infantile onset [the mucopolipidosis I (Cantz *et al.*, 1977)]. This variability in clinical expression of neuraminidase deficiency may be due to different mutations affecting the same locus or

Abbreviation used: MU-AcNeu, sodium (4-methylumbelliferyl *N*-acetyl- α -D-neuraminatate).

different loci. Genetic complementation in skin fibroblast cultures has been applied to this problem but one must first determine the number of molecular forms of human neuraminidase and their respective substrate specificity. Cell hybridization studies indicated that mucopolidosis I and the cherry-red spot syndrome without myoclonus represent mutations at the same locus (Hoogveen *et al.*, 1980). However, there is a disagreement in the literature as to whether the cherry-red spot myoclonus syndrome (with accompanying β -galactosidase deficiency) mutation is allelic to the mucopolidosis I mutation (Kato *et al.*, 1979) or represents a totally different mutation involving an activator factor of both β -galactosidase and neuraminidase (Hoogveen *et al.*, 1980).

To understand the biochemical bases of neuraminidase-deficiency syndromes, it is necessary to purify the enzyme in sufficient quantities for isoenzyme separation and complete physicochemical characterization. Fresh placenta is a convenient and abundant source of human enzyme. In this paper, we report on some kinetic and physical properties of placental neuraminidase and on the presence of at least three molecular forms.

Materials and methods

Materials

MU-AcNeu was synthesized by the method of Potier *et al.* (1979a). Bovine serum albumin (Cohn's fraction V), Triton X-100, fetuin and *N*-acetylneuraminyl-D-lactose were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Octyl β -glucoside was obtained from Calbiochem, La Jolla, CA, U.S.A., and Sephacryl S-300, catalase (EC 1.11.1.6), thyroglobulin, aldolase (EC 4.1.2.13) and ferritin from Pharmacia Fine Chemicals Co., Montréal, Que., Canada. The G_{DIA} ganglioside was purchased from Supelco, Bellefonte, PA, U.S.A. All other reagents were obtained from Fisher Scientific Co. (Montréal). The *N*-acetyl-2,3-didehydro-2-deoxyneuraminic acid inhibitor of neuraminidase was synthesized by the method of Meindl & Tuppy (1969).

Neuraminidase assay

Neuraminidase activity was assayed at 25°C by the method of Potier *et al.* (1979a) with 0.2 mM MU-AcNeu as substrate. The reaction was stopped with 1 ml of ethanol and the proteins were centrifuged at 2000 g for 10 min. The supernatant (1 ml) was collected and mixed with 0.1 ml of 1 M-NaOH just before reading fluorescence with an Aminco-Bowman model 768-H spectrofluorimeter (excitation, 365 nm; emission, 450 nm). Linear assay conditions were obtained with time (up to 45 min

incubation) and amount of homogenate protein (up to 1.6 mg/ml). Unless otherwise stated, the MU-AcNeu substrate was routinely used for neuraminidase assay.

Neuraminidase activity towards *N*-acetylneuraminyl-D-lactose and fetuin was assayed at 37°C by a modification of the method of Tulsiani & Carubelli (1970) in the presence of 1.3 mM-*N*-acetylneuraminyl-D-lactose or 0.32 mg of fetuin/ml as substrate and 0.25 M-sodium acetate buffer, pH 5. Neuraminidase activity with the G_{DIA} ganglioside as substrate was assayed by the method of Leibovitz & Gatt (1968) in the presence of 0.32 mM- G_{DIA} ganglioside, 0.6% (w/v) Triton X-100 and 0.14 M-sodium acetate buffer, pH 5. The liberated sialic acid was determined by the method of Warren (1959) with *N*-acetylneuraminic acid as standard.

When the substrate solutions were replaced by 0.60 mM-*N*-acetylneuraminic acid in the assay mixtures, there was at least a 98% recovery of the added sialic acid after 12 h incubation in the conditions of the assay. This showed that there was no significant *N*-acetylneuraminic aldolase (EC 4.1.3.3) activity under the conditions of the neuraminidase assay. One unit of neuraminidase activity corresponded to the amount of enzyme that hydrolyses 1.0 μ mol of substrate per min.

Preparation of the homogenate and stabilization of neuraminidase activity

Fresh term placentas were obtained from the Maternity Ward of this hospital. The procedures that involved human tissues were approved by the Ethics Committee on Investigation Involving Human Subjects of l'Hôpital Sainte-Justine. Pieces of 1.5–2 g were cut from the maternal side of placenta and used immediately or stored at –60°C until used. Placental neuraminidase is stable in these storage conditions. When needed, the placenta was washed several times with ice-cold 0.15 M-NaCl and homogenized in deionized water (10%, w/v) with a cold Waring blender for 2 min and then with a Potter-Elvehjem homogenizer fitted with a Teflon pestle for a further 2 min at 0°C. Bovine serum albumin was immediately added to the homogenate to stabilize neuraminidase activity. The homogenate was separated into two fractions by centrifugation at 17 000 g for 15 min. The supernatant fraction contained about 61–75% of total neuraminidase activity as determined with the fluorogenic MU-AcNeu substrate.

Sephacryl S-300 chromatography

The soluble neuraminidase (5 ml) was applied to a Sephacryl S-300 column (2.6 cm $\times$ 45 cm) previously equilibrated with 0.02 M-sodium maleate buffer (pH 6.8)/0.1 M-NaCl. Fractions (4.5 ml) were collected and neuraminidase was assayed. The

column was calibrated with Dextran Blue and with the following standard proteins: thyroglobulin, mol.wt. 669 000; ferritin, 440 000; catalase, 232 000; aldolase, 158 000.

Molecular-weight determination by radiation inactivation

Neuraminidase preparations were made 12 mM with respect to EDTA and 10 mg/ml with respect to bovine serum albumin and freeze-dried in 0.2 ml portions in 1.5 ml capped plastic tubes. The freeze-dried powders were flushed with N₂ and irradiated at room temperature (22°C) for different intervals of time up to 20 h in a Gammacell 220 apparatus (Atomic Energy of Canada, Ottawa, Ont., Canada) equipped with a ⁶⁰Co source. The Gammacell 220 was calibrated with enzymes of known radiation inactivation behaviours as previously described (Beauregard *et al.*, 1980; Nguyen *et al.*, 1980). The dose rate was 0.64 Mrad/h. After irradiation, freeze-dried powders were taken up in 0.1 ml water and neuraminidase activity was assayed at least in triplicate.

The molecular weight is obtained from the equation:

$$M_r = 6.4 \times 10^5 / D_{37}$$

where D_{37} is the dose required to inactivate the enzyme to 37% of its initial activity.

Determination of apparent Michaelis–Menten constant

K_m was determined under standard assay conditions with the fluorogenic MU-AcNeu substrate, except that substrate concentration was varied between 0.08 and 4 mM. The graphical method of Lineweaver & Burk (1934) was used for K_m determination.

Protein determination

Protein concentration was determined by the colorimetric method of Bradford (1976), with bovine serum albumin as standard.

Results

Stabilization of neuraminidase activity

The neuraminidase activity is labile at 0°C (half-life, 2.8 h) in crude placental homogenates. Various compounds and pH conditions were tested to stabilize the enzyme (Table 1). CaCl₂ and EDTA were more effective at 12 mM concentration and albumin at 10 mg/ml. The stability of neuraminidase was dependent on pH, being maximum between pH 4.2 and 5.0, with half-maximum values at pH 3.4 and 5.8. Adjusting the placenta homogenate to pH 4.2–5.0 was the most effective way of protecting the enzyme activity. CaCl₂ or albumin was routinely

Table 1. *Stability of placental neuraminidase under different conditions at 0°C*

Homogenate and supernatant fractions were prepared as described in the Materials and methods section, mixed with indicated compounds or buffer, pre-incubated at 0°C for various periods of time and then assayed under standard conditions.

	Half-life (h)
Homogenate + H ₂ O	2.8
Homogenate + 12 mM-CaCl ₂	9.8
Homogenate + 12 mM-EDTA	8.8
Homogenate + albumin (10 mg/ml)	5.0
Supernatant + 12 mM-EDTA	30.0
+ albumin (10 mg/ml)	
Supernatant + 0.05 M-sodium acetate buffer, pH 5	91.2

Table 2. *Effect of various substances and sonication on placental neuraminidase activity*

Neuraminidase (1.6×10^{-5} units) was incubated for 15 min at 0°C in 0.2 M-acetate buffer, pH 4.2, in the presence of the compounds indicated in the Table. After incubation, enzyme activity was determined by the addition of substrate and further incubation at 25°C for 45 min.

	Activity (%)
CaCl ₂ (12 mM)	111
EDTA (12 mM)	77
<i>N</i> -Acetyl-2,3-didehydro-2-deoxyneuraminic acid (0.1 mM)	13
NaCl (0.4 M)	87
KSCN (0.4 M)	16
NaClO ₄ (0.3 M) + 0.05 M-sodium acetate buffer, pH 5	20
Octyl β -glucoside (0.01 M) + CaCl ₂ (12 mM)	114
Triton X-100 (0.05%, w/v) + CaCl ₂ (12 mM)	162
Triton X-100 (2%, w/v) + albumin (5 mg/ml)	112
Sonication for 60 s	60

added to the homogenate to ensure linear assay conditions for neuraminidase activity with incubation time (up to 45 min at 25°C).

The effects of various substances or sonication on placental neuraminidase activity are given in Table 2. CaCl₂, octyl β -glucoside and Triton X-100 activated the enzyme, whereas EDTA, *N*-acetyl-2,3-didehydro-2-deoxyneuraminic acid, NaCl, the chaotropic agents KSCN and NaClO₄ and sonication were all inhibitory or denatured the enzyme. The most effective inhibitor was *N*-acetyl-2-deoxy-2,3-didehydroneuraminic acid, for which a K_i value of $1.6 \mu\text{M}$ was determined by the method of Dixon (1953).

In the presence of 10 mg of albumin/ml, about

61–75% of the placental neuraminidase activity was recovered in the supernatant after centrifugation at 17000 *g* for 15 min. This value decreases to 18–24% when the homogenate is centrifuged at 105000 *g* for 30 min. The presence of CaCl_2 or EDTA or adjusting the pH of the homogenate to 5 resulted in the precipitation of neuraminidase activity. Triton X-100 and octyl β -glucoside were not effective in solubilizing the enzyme in the presence of CaCl_2 or preventing the precipitation of the enzyme, but they rendered the enzyme unstable. Similar results were obtained with NaCl, the chaotropic agents NaClO_4 and KSCN and with sonication.

Evidence for the presence of two neuraminidase components in the placenta

When placental neuraminidase was inactivated at 25°C, a biphasic denaturation curve was obtained (Fig. 1). The thermolabile component (A) had a half-life of 21 min, whereas the more stable component (B) had a half-life of 85 min. Thermal

Fig. 1. Thermodenaturation of placental neuraminidase at 25°C

Supernatant and sediment fractions, obtained by centrifugation of the homogenate at 17000 *g* for 15 min, were pre-incubated at 25°C in the presence of 0.05 M-sodium maleate buffer (pH 6.8)/0.1 M-NaCl, containing 10 mg of bovine serum albumin/ml for different intervals of time and neuraminidase activity was then assayed as described in the Materials and methods section.

denaturation of the supernatant and sedimentable fractions of the homogenate in 10 mg of albumin/ml revealed that the thermolabile A component is associated with the supernatant fraction, whereas the thermostable B component is associated with the sedimentable fraction together with some thermolabile neuraminidase (about 20% of total activity) (Fig. 1). The A and B components had similar pH-activity curves (Fig. 2) and K_m values, 0.10 mM for the A component and 0.06 mM for the B component with the MU-AcNeu substrate.

The rather wide pH-activity curve of neuraminidase with a maximum at pH 4.0–4.4 and a shoulder at pH 5.0 (Fig. 2) suggested that the enzyme may be heterogeneous, representing species with different pH optima as previously shown in human leucocytes (Nguyen *et al.*, 1980). We thus tested this possibility by denaturing the enzyme at 25°C (pH 6.8) and assaying enzyme activity at both pH 4.2 and 5.0. Identical denaturation curves were obtained at both pH values for enzyme assay, suggesting that, if several enzyme components do exist, they have similar denaturation curves (results not shown).

Gel filtration of neuraminidase

The soluble A component was applied to a Sephacryl S-300 column equilibrated with 0.02 M-sodium maleate buffer (pH 6.8)/0.1 M-NaCl. All neuraminidase activity was eluted as a single peak near or at the void volume (results not shown). These results suggest that neuraminidase A exists in an aggregated form, is still bound to high-molecular-weight membrane components or is a highly asymmetric protein.

Fig. 2. pH-activity curves of neuraminidase in the supernatant (●) and sediment (○) fractions of a placenta homogenate

Table 3. *Neuraminidase activity in the placenta*

Substrate	10 ⁶ × Neuraminidase activity (units/g of placenta)	
	Supernatant (A)	Sediment (B)
4-Methylumbelliferyl <i>N</i> -acetyl- α -D-neuraminatate	171 ± 37*	117 ± 29
<i>N</i> -Acetylneuraminyl-D-lactose	28, 30	56, 62
G _{D1A} ganglioside	34 ± 3	93 ± 14
Fetuin	<0.05	<0.05

* Mean ± S.D. of at least three determinations.

Fig. 3. *Radiation inactivation of neuraminidase in the supernatant fraction of placenta*

Bars indicate mean and range of at least triplicate determinations and the straight line was fitted by the least-squares method.

Molecular-weight determination

Radiation inactivation of the soluble neuraminidase A component is shown in Fig. 3. Similar results were obtained with the sedimentable B fraction (results not shown). The molecular weight of the A component (67000 ± 4500) is higher than that of B (63000 ± 6700), but the difference is not statistically significant (Student's *t* test, $P > 0.05$).

Neuraminidase activity towards various natural substrates and competition between substrates

The supernatant and sedimentable fractions of placenta were assayed for neuraminidase activity with a variety of natural substrates in addition to MU-AcNeu (Table 3). To get sufficient activities with the natural substrates, neuraminidase in both fractions was precipitated at pH 5 and the enzyme was resuspended in one-tenth of the initial volume.

Neuraminidase activity with MU-AcNeu as substrate was higher in the supernatant than in the sedimentable fraction, whereas the activity with the *N*-acetylneuraminyl-D-lactose was higher in the sedimentable fraction. About 75% of neuraminidase activity with the G_{D1A} ganglioside substrate was sedimentable. The results suggest heterogeneity of placental neuraminidase as shown by the variable distribution of enzyme activity between supernatant and sediment with the various substrates.

The inhibitory effect of the G_{D1A} ganglioside and *N*-acetylneuraminyl-D-lactose was tested on MU-AcNeu hydrolysis by placental neuraminidases A and B. No inhibition by the G_{D1A} ganglioside (1 mM) was observed but the *N*-acetylneuraminyl-D-lactose (2 mM) inhibited both neuraminidases A and B by 48–56%. These preliminary results suggest that neuraminidases A and B do not bind the G_{D1A} ganglioside and that this glycolipid may be hydrolysed by a different enzyme. In contrast, the neuraminidases A and B that hydrolyse the fluorogenic substrate also bind and probably hydrolyse the *N*-acetylneuraminyl-D-lactose substrate.

Discussion

In crude homogenates, placental neuraminidase is highly unstable but can be stabilized by the addition of CaCl₂, EDTA and bovine serum albumin, but the most effective way was by adjusting the homogenate to pH 5 (Table 1). However, like CaCl₂ and EDTA, this last treatment causes precipitation of proteins and neuraminidase activity that cannot be prevented by addition of Triton X-100, octyl β -glucoside, KSCN or NaClO₄. Only albumin stabilizes without causing precipitation.

In the presence of albumin, it appears that the soluble neuraminidase after centrifugation at 17000 *g* for 15 min or at 105000 *g* for 30 min is aggregated or bound to membranous debris or highly asymmetric since it was excluded entirely from a Sephacryl S-300 column (exclusion limit about 1.5×10^6 for globular proteins), whereas the

molecular weight obtained by radiation inactivation was 67000. We previously reported a molecular weight of 240000–203000 for the human leucocyte neuraminidase, using the radiation inactivation method (Nguyen *et al.*, 1980). Kempner & Schlegel (1979) and Kempner *et al.* (1980) suggested that the radiation inactivation method is sensitive to the functional unit (the minimal structural assembly necessary for a given biological activity) rather than to the structural size of the molecule, and to the degree of coupling between subunits that would allow energy transfer (Kempner & Schlegel, 1979; Beauregard *et al.*, 1980). Our data do not permit a definitive conclusion but it is possible that the leucocyte neuraminidase is composed of three or four equal subunits of a molecular weight similar to that found for the placental neuraminidase. Alternatively, the leucocyte and placental neuraminidase could exist as two different enzymes under separate genetic control. We previously found a molecular weight of 56000 for the rat liver lysosomal and cytosolic neuraminidases close to that of the placental enzyme (Beauregard & Potier, 1982).

Two neuraminidase components, A and B, were characterized in the human placenta using the fluorogenic MU-AcNeu substrate. The A component is thermolabile and soluble when the homogenate is centrifuged at 17000g for 15 min in the presence of 10 mg of bovine serum albumin/ml, whereas the B form is more stable and is sedimentable (Fig. 1). These two forms, however, have similar molecular weights and kinetic properties (pH-activity curves and K_m) (Fig. 2), and both are able to bind *N*-acetylneuraminyl- β -lactose but not the G_{DIA} ganglioside. Two neuraminidase forms differing in their thermolability were previously reported in cultured skin fibroblasts (Potier *et al.*, 1979b), human leucocytes (Nguyen *et al.*, 1980) and mouse liver (Potier *et al.*, 1979c). In cultured skin fibroblasts and in leucocytes of patients with neuraminidase deficiency (sialidosis) both A and B forms were deficient (Potier *et al.*, 1979b; Nguyen *et al.*, 1980), indicating that they are closely related from the genetic point of view. It is possible that the two forms represent conformers, or that one form is the precursor or a hydrolytic degradation product of the other. Okada *et al.* (1981) presented evidence for the presence of a neuraminidase active on colominic acid distinct from that hydrolysing $\alpha 2 \rightarrow 3$ and $\alpha 2 \rightarrow 6$ *N*-acetylneuraminyl- β -lactose in human placenta. The relationship of this $\alpha 2 \rightarrow 8$ neuraminidase to the A and B forms described in this paper is not known since the MU-AcNeu substrate has no linkage specificity.

Several lines of evidence suggest that the neuraminidase active towards glycolipids and that hydrolysing sialic acid residues from glycoprotein are two different enzymes. Wenger *et al.* (1978) and

Cantz & Messer (1979) found normal ganglioside neuraminidase activity in cultured skin fibroblasts of sialidosis patients with a glycoprotein sialidase deficiency. Distinct properties of the two enzymes were also reported (different stability and different effect of inhibitors) in cultured skin fibroblasts (Caimi *et al.*, 1979), horse liver (Sander *et al.*, 1979) and human liver, kidney and brain (Corfield *et al.*, 1981; Veh & Sander, 1981). In addition, Zeigler & Bach (1981) recently reported that the glycoprotein neuraminidase is mainly localized in the lysosomal fraction, whereas the ganglioside neuraminidase is in the plasma-membrane fraction of cultured skin fibroblasts. The placenta has activity towards MU-AcNeu, *N*-acetylneuraminyl- β -lactose and the G_{DIA} ganglioside substrates (Table 3). Competition experiments between substrates indicate that both A and B forms assayed with the artificial MU-AcNeu substrate are probably active on the *N*-acetylneuraminyl- β -lactose but do not bind the G_{DIA} ganglioside. This preliminary result suggests that in the placenta, as well as in many other tissues, this glycolipid may be hydrolysed by a different enzyme.

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