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Differential effect of various inhibitors on four types of rat sialidase

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The inhibitory effect of various compounds on the activities of four types of rat sialidase was investigated. 2-Deoxy-2,3-dehydro-*N*-acetylneuraminic acid and *N*-acetylneuraminic acid were competitive inhibitors for the sialidases. The former was effective against cytosolic sialidase and intralysosomal sialidase more than two membrane-associated sialidases I and II, the latter being a much weaker inhibitor. A heavy metal ion such as Cu^{2+} (1 mM) and thiol-modifying 4-hydroxymercuribenzoate (50 μM) caused complete inhibition of the activities of cytosolic sialidase and membrane sialidase I, while no decrease in the activities of intralysosomal sialidase and membrane sialidase II was observed. When 4-nitrophenyloxamic acid and siastatin B, inhibitors of bacterial sialidases, and synthetic thioglycoside GM3 analogue Neu5Acα-s-(2-6)Galβ(1-4)Glcβ(1-1) ceramide, an inhibitor of influenza virus sialidase, were tested, they did not affect any activity of the rat sialidases. By the differential effect of these inhibitors, the four types of rat sialidase could be discriminated from one another and furthermore from viral and bacterial sialidases.

Keywords: rat sialidase, sialidase inhibitor

Abbreviations: Neu5Ac, *N*-acetylneuraminic acid; Neu5Ac2en, 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid; 4MU-Neu5Ac, 4-methylumbelliferyl-α-*N*-acetyl-D-neuraminic acid.

Introduction

We have previously demonstrated that rat liver contains at least four types of sialidase differing in subcellular location and in catalytic and immunological properties. They are intralysosomal [1], cytosolic [2] and membrane-associated sialidases I and II [3, 4]. Membrane sialidase I is located mainly in the plasma membrane and membrane sialidase II in the lysosomal membrane. Rat brain also possesses the four types of sialidase which have been proved to be identical to the respective enzymes of rat liver in every aspect [3]. Intralysosomal sialidase shows narrow substrate specificity and only oligosaccharides, glycopeptides and a synthetic substrate, 4-methylumbelliferyl-*N*-acetylneuraminic acid (4MU-Neu5Ac) have been shown to be hydrolysed [1]. Sialidase found in the cytosol is also capable of desialylating glycoproteins and gangliosides at near neutral pH [2]. These two sialidases are distinct from membrane-associated sialidases in that the latter requires detergents for solubilization and hydrolyses gangliosides preferentially. Membrane sialidase I hydrolyses only gangliosides, whereas membrane sialidase II also acts on oligosaccharides, glycoproteins and 4MU-Neu5Ac [3]. Although the finding that these sialidases are distinct proteins suggests that each one plays a unique role depending on its unique subcellular location and substrate specificity, no evidence clarifying the physiological function of the sialidases can be offered at present.

Inhibitors for sialidases of different origin have been found to be useful tools in characterization of the enzymes and in elucidation of their biological functions. Of those inhibitors reported so far [5, 6], we employed seven compounds to test their influence on the four types of sialidase and whether they can be used to discriminate the different sialidase molecules. Here we report the results of comparative inhibition studies on *N*-acetylneuraminic acid (Neu5Ac) [7, 8], 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid (Neu5Ac2en) [8, 9], Cu^{2+} [10, 11], and 4-hydroxymercuribenzoate [11], on three synthetic compounds, 4-nitrophenyloxamic acid [8, 13], and a thioglycoside GM3 analogue Neu5Acα-s-(2-6)Galβ(1-4)Glcβ(1-1)ceramide [14], and on

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siastatin B [15], a compound from the culture filtrate of streptomyces.

Materials and methods

Substrate and inhibitors

4MU-Neu5Ac was purchased from Nakarai (Japan). Bovine mixed gangliosides, bovine colostrum sialyllactose and fetuin (type IV) were from Sigma (USA). GM3 was isolated from dog erythrocytes. GM3 labelled in the Neu5Ac moiety was synthesized from lactosylceramide (Sigma) and CMP-[^{14}C]Neu5Ac (New England Nuclear, USA) using the Golgi fraction of rat AH-109A ascites hepatoma as the source of GM3 synthase and diluted with nonradioactive GM3 to give a final radioactivity of 1000 counts min^{-1} $nmol^{-1}$. The procedure has been described in detail previously [16].

Neu5Ac and Neu5Ac2en were obtained from Sigma. 4-Nitrophenyloxaminic acid was a generous gift from Dr R. Schauer, Christian-Albrechts-Universität, Kiel, Germany. Thioglycoside GM3 analogue Neu5Acα-s-(2-6)Galβ(1-4)-Glcβ(1-1)ceramide was synthesized as described previously [17]. Siastatin B was purified from the culture filtrate of streptomyces [18]. 4-Hydroxymercuribenzoate was purchased from Sigma, and other thiol reagents were from Wako (Japan).

Sialidase preparations

Cytosolic sialidase was partially purified from rat liver or from rat skeletal muscle by the procedure described previously [2, 4] until the CM-cellulose step. Intralysosomal sialidase was prepared from rat liver as described previously [1]. The membrane sialidases I and II of rat brain were purified according to the procedures described previously [3] except that steps 5–7 were omitted. The membrane sialidase I was also prepared from the plasma membrane fractions isolated from rat liver as described [4].

Sialidase assay

The standard assay mixture contained 100–200 nmol (as bound sialic acid) of substrate, appropriate amount of inhibitor, 0.2 mg bovine serum albumin, 15 μmol sodium acetate, pH 5.5 or pH 4.5, and enzyme (7 to 10 units) in a final volume of 0.2 ml. The buffer of pH 5.5 was used for cytosolic sialidase and of pH 4.5 for the other three sialidases, unless stated otherwise. The substrates employed here were 4MU-Neu5Ac, fetuin and bovine mixed gangliosides for cytosolic sialidase, 4MU-Neu5Ac for intralysosomal sialidase and gangliosides for membrane sialidases I and II, respectively, since they were hydrolysed effectively by the respective enzymes [1–3]. With gangliosides as substrate, the assay mixture contained 0.1 mg sodium cholate for cytosolic sialidase and 0.1 mg Triton X-100 for membrane sialidases. After incubation at 37 °C for 1 h, the sialic acid released was determined by the thiobarbituric acid method of Warren [19], and 4-methylumbelliferone released using a spectrofluorometer [2].

When the inhibitory effect of Neu5Ac was tested with the ganglioside substrate, 10 nmol GM3 ^{14}C labelled in the Neu5Ac moiety was included as the substrate in a half volume of the reaction mixture for non-radioactive substrate. The mixture was incubated at 37 °C for 1 h, and the reaction was terminated by adding 1 ml 5% (*w/v*) trichloroacetic acid containing 1% (*w/v*) phosphotungstic acid. The sialic acid released was then determined as described previously [16]. One unit of sialidase was defined as the amount of enzyme which catalysed the release of 1 nmol sialic acid in 1 h.

Results and discussion

Five specific inhibitors of sialidase, Neu5Ac2en, Neu5Ac, 4-nitrophenyloxamic acid, siastatin B and a thioglycoside GM3 analogue Neu5Acα-s-(2-6)Galβ(1-4)Glcβ(1-1)-ceramide, were examined for inhibitory activity first by using cytosolic sialidase prepared from rat skeletal muscle or from rat liver. 4MU-Neu5Ac was used as substrate except when testing 4-nitrophenyloxamic acid, in which case sialyllactose was used, since this inhibitor has been reported to cause quenching with 4MU-Neu5Ac substrate [12]. Of the five inhibitors, only Neu5Ac and Neu5Ac2en were found to affect cytosolic sialidase. When heavy metal ions such as Cu^{2+} and Hg^{2+} and thiol-modifying 4-hydroxymercuribenzoate were tested, they caused a marked inhibition of cytosolic sialidase.

To determine if different substrates and pHs influence the inhibitory activities, the activity of cytosolic sialidase of skeletal muscle was measured at increasing concentration of inhibitors using 4MU-Neu5Ac, fetuin or gangliosides as substrate at pH 5.5 or pH 4.5 (Fig. 1). With ganglioside substrate, the inhibitory effect of Neu5Ac2en and Neu5Ac was less pronounced than with fetuin or 4MU-Neu5Ac. The lower effectiveness is not due to sodium cholate added in the assay with ganglioside substrate, since it did not alter even when sodium cholate was added to the reaction mixture with fetuin or 4MU-Neu5Ac substrate; but is probably due to their micellar structure, as suggested by the experiment showing the inhibitory activities measured with α(2-3) sialyllactose were higher than those with GM3, even though the former is just the sugar moiety of the latter. Unlike these inhibitors, Cu^{2+} and 4-hydroxymercuribenzoate affected similarly the sialidase activity assayed with every substrate. Shifting pH 5.5 to pH 4.5 in the reaction resulted in little change in the inhibitory activities as demonstrated with Neu5Ac2en and 4-hydroxymercuribenzoate (Fig. 1(a, b)). Replacing the detergent, sodium cholate, by TritonX-100 in the assay with ganglioside substrate had no influence on the inhibitory activities (data not shown). Cytosolic sialidase

Table 1. Comparative studies on inhibition of four types of rat sialidase by four compounds.

Inhibitors (mM)	*Substrate*	*Intralysosomal sialidase*	*Cytosolic sialidase*	*Membrane sialidase*	
				I	*II*
			Residual activity (%)		
Neu5Ac2en (0.1 mM)	4MU-Neu5Ac	47	2	–	65
	Gangliosides	–[a]	30	82	78
Neu5Ac (2 mM)	4MU-Neu5Ac	50	38	–	95
	Gangliosides	–	68	100	100
4-Hydroxymercuribenzoate (10 μM)	4MU-Neu5Ac	136	0	–	97
	Gangliosides	–	0	28	100
Cu^{2+} (0.5 mM)	4MU-Neu5Ac	100	0	–	ND[b]
	Gangliosides	–	0	45	100

[a] Due to the inability to hydrolyse the substrate by the sialidase indicated, the values were not determined.
[b] Not determined.

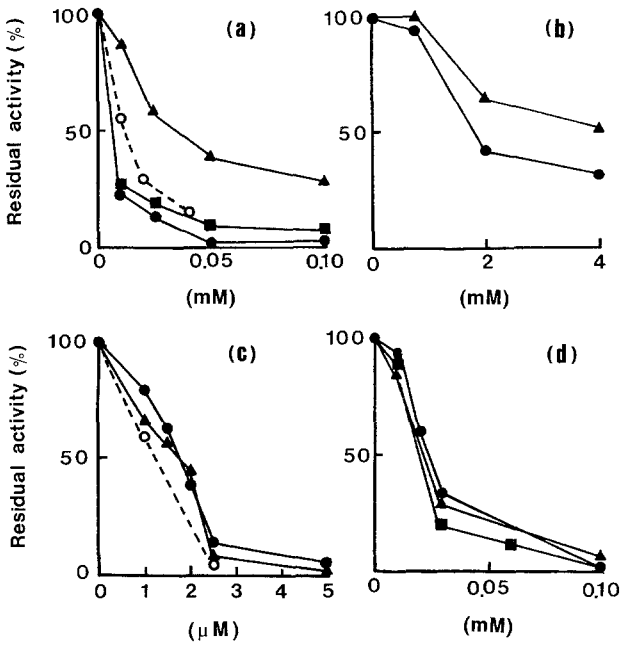

Figure 1. Inhibition of cytosolic sialidase by (a) Neu5Ac2en, (b) Neu5Ac, (c) 4-hydroxymercuribenzoate, and (d) $CuCl_2$ using ●, ○, 4MU-Neu5Ac, ■, fetuin or ▲, gangliosides as substrate. For ganglioside substrate, bovine mixed gangliosides were used in (a), (c) and (d), and [^{14}C]GM3 in (b). The assay was conducted mostly at pH 5.5 and exceptionally at pH 4.5 (○).

prepared from rat liver gave the same results as the muscle sialidase.

Under the appropriate conditions demonstrated above, the inhibitory effect of those compounds against the four types of sialidase was compared as shown in Table 1. Based on the substrate preference of these enzymes, the inhibition of the activities of cytosolic sialidase and membrane sialidase II was measured with the two substrates, 4MU-Neu5Ac and gangliosides, and intralysosomal sialidase and membrane sialidase I were assayed with 4MU-Neu5Ac and with gangliosides, respectively. Neu5Ac2en affected all four types of sialidase and inhibited cytosolic sialidase most effectively of the four sialidases. Neu5Ac was also active against cytosolic and intralysosomal sialidases at concentrations as high as 10^{-3} M, but inactive against the two membrane sialidases. The most potent inhibition by Cu^{2+} was observed with cytosolic sialidase and somewhat less with membrane sialidase I. However, no inhibition was obtained using the two lysosomal sialidases, viz., intralysosomal sialidase and membrane sialidase II. Hg^{2+} caused inhibition similar to that seen by Cu^{2+} (data not shown). Thiol-modifying reagents also affected sialidase activity. 4-Hydroxymercuribenzoate strongly inhibited the activities of cytosolic sialidase and membrane sialidase I, while the reagent was not an inhibitor for the two lysosomal sialidases; intralysosomal sialidase was slightly activated rather than inhibited by the reagent. *N*-Ethylmaleimide and 5,6′-dithiobis(2-nitrobenzoic acid) were effective only at a much higher concentration (5 mM) than was used with 4-hydroxymercuribenzoate. The inhibition by either the thiol reagent or Cu^{2+} was reversed by subsequent addition of dithiothreitol (2 mM) or EDTA (5 mM), respectively. These results, together with the inhibition caused by heavy metal ions, suggest that free sulfhydryl groups are essential for the catalytic activities of cytosolic sialidase and membrane sialidase I. It should be noted that the results obtained with brain membrane sialidase I were essentially the same as those with the enzyme from liver plasma membrane, independent of tissue source of sialidase.

In contrast to the above compounds, 4-nitrophenyl oxamic acid, siastatin B and a thioglycoside GM3 analogue, Neu5Acα-s-(2-6)Galβ(1-4)Glcβ(1-1) ceramide were ineffective against any of the rat sialidases at the concentration shown. 4-Nitrophenyl oxamic acid (3 mM) exhibited no inhibitory activity, whereas the K_i value for *Vibrio cholerae*

Table 2. K_i values for Neu5Ac2en and Neu5Ac obtained with four types of rat sialidase.

Inhibitor	*Substrate*	*Intralysosomal sialidase*	*Cytosolic sialidase*	*Membrane sialidase*	
				I	*II*
			(M)		
Neu5Ac2en	4MU-Neu5Ac	3.0×10^{-5}	1.2×10^{-6}	–[a]	ND[b]
	GM3	–	3.3×10^{-5}	2.8×10^{-4}	2.3×10^{-4}
Neu5Ac	4MU-Neu5Ac	2.4×10^{-3}	1.3×10^{-3}	–	ND

[a] Due to the inability to hydrolyse the substrate by the sialidase indicated, the values were not determined.
[b] Not determined.

sialidase was described to be 0.58 mM [13]. At the concentration *Clostridium perfringens* sialidase was inhibited completely (100 and 500 μg/ml), siastatin B failed to inhibit the rat sialidases; the GM3 analogue had no significant effect on the sialidases even though a 20-fold higher concentration (0.3 mM) than the K_i value for influenza virus sialidase was tested.

To examine the mechanism of inhibition of Neu5Ac2en or Neu5Ac, cytosolic sialidase, which was found most sensitive to the inhibitors, was assayed with increasing concentrations of 4MU-Neu5Ac substrate in the presence or absence of the inhibitors. Figure 2 (a, b) shows that they were competitive inhibitors, as reported previously for bacterial and viral sialidases [8, 9, 13]. The K_i values for Neu5Ac2en and Neu5Ac were calculated from Dixon plots [20] as shown in Fig. 2 (c, d). The values obtained with the four types of sialidase were compared in Table 2. Marked differences were found in the values for Neu5Ac2en: the K_i for intralysosomal sialidase was about 20-fold higher than that for cytosolic sialidase with 4MU-Neu5Ac substrate, and those for two membrane sialidases were 10 times that of cytosolic enzyme when gangliosides were substrate. The K_m values were 0.11 mM for cytosolic sialidase and 0.17 mM for intralysosomal sialidase with 4MU-Neu5Ac, and the values with GM3 were 0.59 mM for cytosolic sialidase, 0.13 mM for membrane sialidase I and 0.022 mM for membrane sialidase II. These results indicate that under the appropriate assay conditions the sialidase specific inhibitors such as Neu5Ac and Neu5Ac2en can discriminate cytosolic and intralysosomal sialidases from the two membrane sialidases, and that heavy metal ions and thiol-modifying reagents, on the other hand, can discriminate between the non-lysosomal enzymes and the two lysosomal enzymes. Consequently, the four types of rat sialidase are distinguishable from one another by the use of two inhibitors, either Neu5Ac2en or Neu5Ac and Cu^{2+} or 4-hydroxymercuribenzoate. In comparison with sialidases of bacterial and viral origins, the degree of inhibition of cytosolic sialidase by Neu5Ac2en or Neu5Ac was comparable to those obtained with sialidases of non-animal origin: in previous

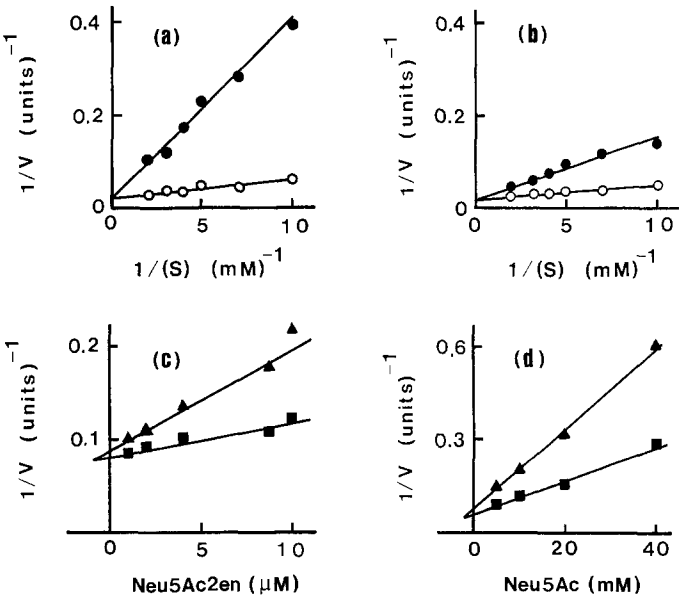

Figure 2. The effects of Neu5Ac2en and Neu5Ac on the hydrolysis of 4MU-Neu5Ac by cytosolic sialidase. The sialidase activity was assayed ○ in the absence, and ● in the presence, of (a) 0.04 mM Neu5Ac2en or (b) 10 mM Neu5Ac (b). Dixon plots were obtained from the data on the effects of (c) Neu5Ac2en and (d) Neu5Ac on the sialidase activity at the different concentrations of 4MU-Neu5Ac (▲, 0.15 mM; ■, 0.30 mM).

observations, K_i values were calculated as 4.88 mM for Neu5Ac with *Vibrio cholerae* sialidase [21] and 0.79 μM for Neu5Ac2en with influenza virus sialidase [9]. Cytosolic sialidase, however, was not affected by 4-nitrophenyloxamic acid, siastatin B and a GM3 thioanalogue as described above, which are potent inhibitors for bacterial and viral sialidases, indicating that the enzyme is clearly discriminated from the sialidases of bacterial and viral origins in the behaviour towards the inhibitors. In this connection, it is interesting to note that cytosolic sialidase appears to be closely related to a sialidase from *Trypanosoma brucei* which has been described to be inhibited by Neu5Ac2en and 4-hydroxymercuribenzoate but not by 4-nitrophenyl oxamic acid [12].

As expected from the previous studies on catalytic properties and antigenic specificity, the four types of sialidase were demonstrated to behave differently towards the inhibitors tested, presenting further evidence that the enzymes are distinct from one another. The results also support the idea that the structure of the active site may be different in these sialidases. The structures and functions of these enzymes remain to be elucidated.

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