THE ANOMERIC CONFIGURATION OF *N*-ACETYLNEURAMINIC ACID RELEASED BY THE ACTION OF *VIBRIO CHOLERAE* NEURAMINIDASE

Leif HOLMQUIST and Börje ÖSTMAN
National Defence Research Institute, Departments 4 and 2,
S-172 04 Sundbyberg 4, Sweden

Received 23 October 1975

1. Introduction

A mechanism for the action of *Vibrio cholerae* neuraminidase, which releases α-ketosidically bound *N*-acetylneuraminic acid, has recently been suggested [1]. The enzymatic cleavage may be formulated:

$$E + S \rightleftarrows E \sim S \xrightarrow{H_3O^+} E \sim P_1 + P_2 \xrightarrow{H_2O}$$

$$E \sim P_1 - OH + H_3O^+ + P_2 \rightleftarrows E + P_1 - OH +$$

$$H_3O^+ + P_2$$

In the proposed reaction sequence, a cyclic oxocarbonium ion of *N*-acetylneuraminic acid, fig.1, is assumed to react with water to produce the β-anomer directly or by the rapid anomerization of an initially formed α-anomer.

P_1 represents the cyclic oxocarbonium ion, P_1–OH the free *N*-acetylneuraminic acid, P_2 the released aglycone, E the enzyme and S the substrate. As β-ketosides of *N*-acetylneuraminic acid are not cleaved by neuraminidase, it was anticipated that they have no or only a weak affinity for the enzyme. A β-*N*-acetylneuraminic acid–enzyme complex should thus rapidly dissociate, thereby allowing a new substrate molecule to reach the catalytic site. The inhibitory effect of *N*-acetylneuraminic acid [2] on the enzyme may be explained by the presence of a limited amount of the α-anomer in equilibrium with the β-anomer.

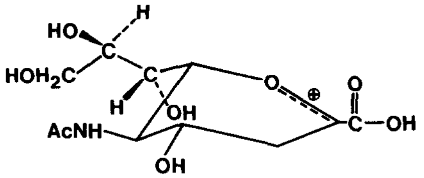

Fig.1. Structure of the cyclic oxocarbonium ion of *N*-acetylneuraminic acid.

A ^{13}C n.m.r. study of *N*-acetylneuraminic acid has recently shown that the nonulosaminic acid in water [3] produces a spectrum typical for a single anomeric form. The β-configuration was assigned by analogy with the corresponding resonances for *N*-acetylneuraminic acid and its methyl β-ketoside.

In the present paper the ^{13}C n.m.r. spectra of *N*-acetylneuraminic acid and its methyl ester and their specific optical rotation properties in methylsulfoxide and water are compared. The results afford additional evidence that *N*-acetylneuraminic acid exists predominantly in the β-configuration in water. Further the affinity of *Vibrio cholerae* neuraminidase for *N*-acetylneuraminic acid bound to Sepharose as β-ketoside has been studied.

2. Material and methods

2.1. *General methods*

Neuraminidase from *Vibrio cholerae* (glycoprotein *N*-acetylneuraminyl-hydrolase, EC 3.2.1.18) was purchased from Behring-Werke, Marburg; 1 ml containing 500 units (producer's specification). *N*-Acetylneuraminic acid was isolated according to [4] and crystallized twice from acetic acid–water (20:1). Melting points are corrected. Free *N*-acetylneuraminic acid was determined according to Warren [5]. The methyl ester of *N*-acetylneuraminic acid was prepared according to [6]. Optical rotations were determined with a Perkin-Elmer 141 polarimeter using a 10 cm cell. 20 MHz ^{13}C n.m.r. spectra were recorded in 8 mm tubes at 37°C in a Varian CFT 20 spectrometer with proton noise decoupling and with off resonance decouplings for retention of ^{13}C–H couplings. For the shift assignments, dioxane was used as secondary reference for the deuterium oxide solutions and the central band of the dideuteromethyl multiplet as secondary reference for the hexadeutereomethylsulfoxide solutions. The nonulosaminic acid derivatives were analyzed at concentrations of 100–200 mg per ml.

2.2. *Syntheses*

2.2.1. Butoxycarbonylmethyl β-ketoside of tetra-*O*-acetyl-*N*-acetylneuraminic acid

The preparation of the butoxycarbonylmethyl α-ketoside of tetra-*O*-acetyl-*N*-acetylneuraminic acid [7] described earlier, afforded after evaporation of the chloroform layer a hard glass from which the β-anomer was obtained by fractional crystallisation. From a typical preparation, using tetra-*O*-acetyl-*N*-acetylneuraminic acid (1 g) as starting material, about 10 mg pure β-anomer was obtained from the first crop of crystals which crystallized from water–acetone (9:1) at room temperature. After recrystallisation from water–acetone (9:1), the constants were $Mp._{dec.}$ = 198–200°C. $[\alpha]_D^{25} = -7°$ (c = 1.0 methanol). Hydrolysis of the *O*-acetyl groups with 1 M sodium hydroxide for 15 min at room temperature resulted in authentic carboxymethyl β-ketoside of *N*-acetylneuraminic acid [7].

The α-anomer of the peracetylated butoxycarbonylmethyl ketoside of *N*-acetylneuraminic acid crystallized from the mother liquid at +5°C. The yield was 200 mg (18%).

$Mp._{dec.}$ = 174–176°C, (water–acetone, 9:1).

$[\alpha]_D^{25} = -12°$ (c = 1.0 in methanol).

Literature [7]: $Mp._{dec.}$ = 184–186°C, (water).

$[\alpha]_D^{25} = -10°$ (c = 0.5 in methanol).

2.2.2. 2-Aminoethylaminocarbonylmethyl β-ketoside of *N*-acetylneuraminic acid

The butyloxycarbonylmethyl β-ketoside of *N*-acetylneuraminic acid (40 mg) was treated with anhydrous ethylene diamine (0.5 ml) as described for the preparation of the corresponding α-anomer [8]. After treatment with Amberlite IRC 50 (H^+), the β-ketoside was applied to a 1 × 2 cm Dowex-1 × 8 (acetate form) column. Elution with water resulted in a chromatographically pure product. After freeze-drying, the yield was 20 mg (69%).

$[\alpha]_D^{25} = -44°$ (c = 1.0 in water).

2.2.3. Sepharose bound β-ketoside of *N*-acetylneuraminic acid

The 2-aminoethylaminocarbonylmethyl β-ketoside of *N*-acetylneuraminic acid (10 mg) was coupled to Sepharose-4B (2 ml wet gel) activated with cyanogen bromide (300 mg) as previously described for the preparation of Sepharose bound α-ketosides of *N*-acetylneuraminic acid [8].

The amount of matrix-bound *N*-acetylneuraminic acid was estimated to 1.3 mmol/ml wet gel after treatment of the gel (0.4 ml) with 0.2 M hydrochloric acid (0.4 ml) at 80°C for 2 h. The stability of the β-ketosidic linkage and the instability to acid of free *N*-acetylneuraminic acid, probably results in an underestimate of bound *N*-acetylneuraminic acid under these conditions.

2.3. *Column experiments*

A column of β-*N*-acetylneuraminic acid–Sepharose (5 × 5 mm) was equilibrated with 1.0 M Tris-maleate buffer pH 6.40, 0.01 M with respect to calcium chloride at 0°C.

Neuraminidase (0.3 ml, 150 units) was applied to the column and was allowed to penetrate into the gel. After 15 min, two 0.1 ml portions of buffer were added to the column and the enzyme was eluted with the same buffer as above. Fractions of 1 ml were collected at a flow rate of 0.2–0.3 ml/min. A buffered solution of the benzyl α-ketoside of *N*-acetylneuraminic acid (400 μg) in water (25 μl) was added to each fraction. Neuraminidase activity was determined by incubation of the fractions at 37°C for 30 min and 18 h. Samples (200 μl) were removed and released *N*-acetylneuraminic acid was determined according to [5].

Attempted desorption of neuraminidase from the columns was performed by addition of the benzyl α-ketoside of *N*-acetylneuraminic acid to the eluant (400 μg/ml). The effluent was incubated and analyzed for neuraminidase activity as above omitting the addition of substrate.

3. Results and discussion

N-acetylneuraminic acid has been reported [9] to mutarotate in water – the specific optical rotation values, $[\alpha]_D$, changing from about –36° after 6 min, –55° after 13 min to a constant value of –35° after 40 min. In conflict with these findings it has been demonstrated that *N*-acetylneuraminic acid shows no mutarotation in water with a constant specific optical rotation value of –32 ± 2° [10,11].

According to [12], mutarotation of the nonulosaminic acid in methylsulfoxide also occurs, with an initial extrapolated value of $[\alpha]_D = -150°$ to –200°, changing to –115° after 7 min, –37° after 37 min., and at equilibrium –24°. The latter results were interpreted in favor of a β-configuration for the crystalline *N*-acetylneuraminic acid which in solution is in equilibrium with its α-anomer. The β-configuration of crystalline *N*-acetylneuraminic acid has recently been confirmed [13].

In the present investigation, a solution of *N*-acetylneuraminic acid in water or methylsulfoxide yielded ^{13}C n.m.r. spectra, typical for the presence of a single anomeric configuration (table 1). In methylsulfoxide–water, 3:1 the nonulosaminic acid, also behaved as one anomer. No differences in spectra of solutions recorded immediately or after 2 days ageing was observed.

Furthermore, in contrast to previous findings [12] no mutarotation of *N*-acetylneuraminic acid in anhydrous methylsulfoxide or methylsulfoxide–water, 3:1 could be observed. Values recorded after 5 min and after 2 days were $[\alpha]_D^{25} = -39 \pm 2°$, $c = 1.0$ and 0.2 for boths solvents.

In water, *N*-acetylneuraminic acid also failed to mutarotate, showing $[\alpha]_D^{25} = -32 \pm 2°$, $c = 1.0$ in agreement with [10,11]. It has been demonstrated that the methyl ester of *N*-acetylneuraminic acid exists as the β-anomer in methylsulfoxide and in its crystalline form, by ^1H n.m.r. spectrometric [14] and X-ray crystallographic studies [15], respectively. The methyl ester does not mutarotate in water or methylsulfoxide [14] with $[\alpha]_D^{20} = -28°$ $c = 1.0$ and $[\alpha]_D^{20} = -33°$ $c = 1.0$ respectively. Both values were constant over a week.

Table 1
^{13}C Chemical shifts of *N*-acetylneuraminic acid and its methyl ester (p.p.m. from external tetramethylsilane)

	Deuterium oxide		Hexadeutereomethyl-sulfoxide	
Carbon	A	B	A	B
C-1	173.89	172.23	171.51	170.41
C-2	96.07	96.17	94.85	95.11
C-3	39.67	39.50	–[b]	–[b]
C-4	67.39[a]	67.39[a]	65.87	65.72
C-5	52.98	52.92	53.19	53.17
C-6	70.99	70.96	69.99	69.83
C-7	69.07	69.05	69.36	69.24
C-8	71.29	71.19	70.58	70.64
C-9	64.00	63.99	63.84	63.74
CH_3-	22.92	22.91	22.77	22.73
$\overset{\mathrm{O}}{\overset{\|}{\mathrm{C}}}\mathrm{-NH}$	175.65	175.81	172.06	172.14
CH_3O	–	54.32	–	52.46

A = *N*-acetylneuraminic acid (3,5-dideoxy-5-acetamido-D-glycero-D-galacto-nonulosonic acid

B = Methyl ester of *N*-acetylneuraminic acid (Methyl 3,5-dideoxy-5-acetamido-D-glycero-D-galacto-nonulosonate)

[a] Same position as dioxane.

[b] Hidden by solvent peak.

Like *N*-acetylneuraminic acid, the ^{13}C n.m.r. spectra of the methyl ester in water or methylsulfoxide indicated the presence of a single anomeric configuration. The very close agreement of the resonances for corresponding carbon atoms, except C-1, in *N*-acetylneuraminic acid and its methyl ester, (table 1) is consistent with a β-configuration of *N*-acetylneuraminic acid in solution.

In order to examine the affinity of the neuraminidase for β-ketosides of *N*-acetylneuramic acid, the enzyme was applied to a column of Sepharose-bound 2-aminoethylaminocarbonylmethyl β-ketoside of *N*-acetylneuraminic acid (β-*N*-acetylneuraminic acid-Sepharose).

The enzyme was eluted from the column by 0.1 M Tris-maleate buffer, pH 6.40. The elution profile demonstrates a significant but weak affinity of the enzyme for the β-*N*-acetylneuraminic acid–Sepharose conjugate (fig.2).

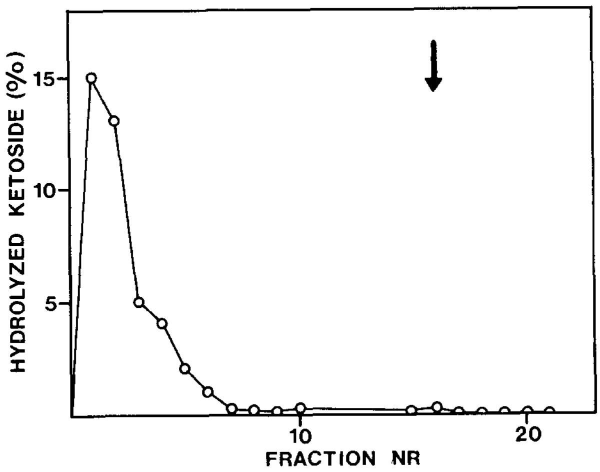

Fig.2. Activity of *Vibrio cholerae* neuraminidase in the effluent from a column of β-*N*-acetylneuraminic acid-Sepharose. Incubation was performed for 30 min. Eluant: 0.1 M Tris-maleate buffer 6.40, 0.01 M with respect to calcium chloride. The arrow denotes the start of the addition of the benzyl α-ketoside of *N*-acetylneuraminic acid. For further details see text.

After completed elution of neuraminidase by the buffer, no more enzyme could be recovered from the column by the addition of the benzyl α-ketoside of *N*-acetylneuraminic acid to the eluant. Recently it was shown that *Vibrio cholerae* neuraminidase was very strongly adsorbed by α-*N*-acetylneuraminic acid–Sepharose type V [8] and to effectively desorb the enzyme from the column it was necessary to add the benzyl α-ketoside of *N*-acetylneuraminic acid to the eluant.

The present results support the assumption that the *N*-acetylneuraminic acid, produced by the enzymatic cleavage of its α-ketosides, leaves the catalytic site of neuraminidase as the β-anomer.

Acknowledgement

The authors are indebted to Professor Klas-Bertil Augustinsson for his interest in this work.

References

[1] Holmquist, L. (1975) FOA Reports 9, No. 3, 1–20.
[2] Mohr, E. (1960) Z. Naturforsch. 15 b, 575–577.
[3] Bhattacharjee, A. K., Jennings, H. J., Kenny, C. P., Martin, A. and Smith, I. C. P. (1975) J. Biol. Chem. 250, 1926–1932.
[4] Zilliken, F. and O'Brien, P. J. (1960) Biochem. Prep. 7, 1–5.
[5] Warren, L. (1959) J. Biol. Chem. 234, 1971–1975.
[6] Kuhn, R., Lutz, P. and Mac Donald, D. L. (1966) Chem. Ber. 99, 611–616.
[7] Holmquist, L. and Brossmer, R. (1972) FEBS Lett. 22, 46–48.
[8] Holmquist, L. (1974) Acta Chem. Scand. B 28, 1065–1068.
[9] Scheinthal, B. M. and Bettelheim, F. A. (1968) Carbohyd. Res. 6, 257–265.
[10] Klenk, E. and Faillard, H. (1954) Hoppe Seyler's Z. Physiol. Chem. 298, 230–238.
[11] Zilliken, F., Braun, G. A. and György, P. (1956) J. Biol. Chem. 63, 394–402.
[12] Kuhn, R. and Brossmer, R. (1959) Chem. Ber. 92, 1667–1671.
[13] Flippen, J. L. (1973) Acta Cryst. B 29, 1881–1886.
[14] Jochims, J. C., Taigel, G., Seeliger, A., Lutz, P. and Driessen, H. E. (1967) Tetrahedron Letters 44, 4363–4369.
[15] Connell, A. M. (1973) Acta Cryst. B 29, 2320–2328.