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CONFIGURATION OF SUBSTRATE AND PRODUCTS OF N-ACETYLNEURAMINATE PYRUVATE-LYASE FROM CLOSTRIDIUM PERFRINGENS

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To characterize the true substrate for aldolase from Clostridium perfringens (optimum pH = 7.2) several experiments were carried out wherein the substrate Neu5Ac was generated in situ at pH 5.4 by the action of sialidase on its substrate Neu5Ac(α,2→3)lactose. The α-anomer formed in this reaction was found to be split by aldolase at this pH into ManNAc and pyruvate. β-Neu5Ac as such was not converted at pH 5.4. However, when it was first mutarotated until the equilibrium mixture α:β = 7.2:92.8 was obtained, it could be split. Inhibition experiments suggested that Neu5Ac was bound to the enzyme in a conformation that strongly resembled that of its α-anomer.
The open chain form of ManNAc which arose after the action of aldolase preferentially formed the α-anomer followed by a fast mutarotation.

N-Acetylneuraminate pyruvate-lyase (aldolase; EC 4.1.3.3) catalyses the degradation of N-Acetylneuraminic acid (Neu5Ac) to pyruvate and N-Acetyl-D-Mannosamine (ManNAc) [1,2]. The enzyme from Clostridium perfringens has been purified to homogeneity. Its molecular weight is 99000 Dalton and it has a pH-optimum of 7.2 [3,4]. The reaction catalysed by the enzyme at this pH is schematically presented in fig. 1.
Regarding the substrate the following observations have been reported. Apparently a covalent bond between C2 of Neu5Ac and the ε-nitrogen atom from a lysine residue in the enzyme is formed [4,5]. It has further been suggested that the unpronated imidazole nitrogen atom of a histidine with a pK of about 6.4 accepts the proton from the hydroxyl group at C-4. Subsequently, ManNAc is split off [4]. The same Schiff's base has been postulated for the interaction of N-Acetyl-3-hydroxy-neuraminic acid with the enzyme [6]. Though never detected, the open form is believed to be an intermediate in the α-β mutarotation of Neu5Ac in analogy to other sugars. Neu5Ac exists only in the pyranose form. In aqueous solution the β-anomer concentration is a factor of ten larger than that of the α-anomer [7,8]. However, crystallized Neu5Ac used in enzyme activity tests contains only the β-anomer.
In order to gain a better insight into the interaction between substrate and enzyme, we compared the suitability of the anomeric forms of Neu5Ac to act as primary substrate. α-Neu5Ac was obtained in situ by the action of sialidase (EC 3.2.1.18) on its substrate Neu5Ac(α,2→3)lactose [8]. Furthermore, we prepared several Neu5Ac derivatives to carry out inhibition experiments.

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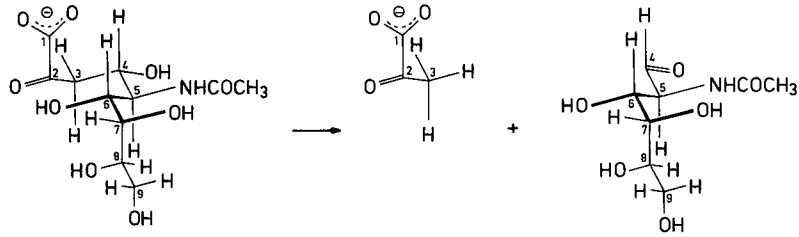

Neu5Ac (open form) pyruvic acid ManNAc (open form)

Fig. 1 Cleavage of Neu5Ac to pyruvic acid and ManNAc

Materials and methods

Aldolase (Clostridium perfringens) was purchased from Sigma (grade III, 1.15 U/mg protein). Sialidase was purchased from Boehringer (0.6 U/mg protein) and further purified by preparative polyacrylamide gel electrophoresis as described earlier [9,10]. Neu5Ac was isolated according to Montreuil [11], further purified on a silica column (eluent: n-butanol/acetic acid/water=2/1/1, v/v) and crystallized twice from water/methanol/ether= 1/10/25, v/v. Neu5Ac(α,2→3)lactose and glycosides from Neu5Ac methyl esters were gifts from Dr G.Strecker and Dr D.J.M.v.d.Vleugel, respectively.

Neu5Ac alditol was prepared by treating 50 mg Neu5Ac in 50 ml destilled water with 200 mg $NaBH_4$ (Baker) for 2 hr at room temperature. The pH was then adjusted to 5 with Dowex 50x8 (100-200 mesh, H^+form). The resin was filtered off and washed twice with 50 ml water. After evaporation of water, the residu was codestilled three times with methanol. The method yielded the alditol quantitatively. It gave a single spot on TLC and as trimethylsilyl derivative one peak on GLC. The identity was checked with GCMS. The α-methyl, α-ethyl and β-methyl glycosides of Neu5Ac methyl ester were saponified by treatment with 10 equivalents of trimethylamine at 4°C overnight, yielding quantitatively the corresponding glycosides. The products were analyzed by TLC, GLC and GCMS. ^{1}H-NMR spectra were recorded on a Bruker HX-360 spectrometer operating in the Fourier transform mode at a probe temperature of 22°C. Enzyme experiments were performed directly in the spectrometer. Substrates and buffer salts were treated several times with D_2O (Merck, 99.75%) at room temperature with intermediate lyophilization. Enzyme preparations were lyophilized once. Chemical shifts are given relative to sodium-4,4-dimethyl-4-silapentane-1-sulphonate (indirectly to internal acetone: 2.225 ppm). 50 scans per spectrum were used (16 K data points; accumulation time: 2 minutes). GLC was carried out on a Varian Aerograph 2740, equipped with a dual flame ionization detector and glass columns (2.00 m x 4.0 mm) packed with 3.8% of SE-30 on Chromosorb W-HP (80-100 mesh). The oven temperature was kept 18 min at 185°C followed by a temperature gradient of 2°/min up to 250°C. The nitrogen flow was 35 ml/min. Trimethylsilylation of the samples was performed with hexamethyldisilazane/ trimethylchlorsilane/pyridine=1/1/5, v/v. GCMS was performed with a combined Carlo-Erba gas chromatograph/Kratos MS80 mass spectrometer. 70 eV mass spectra were recorded using an ion source temperature of 200°C, an accelerating voltage of 3 kV and an ionizing current of 300 μA. The glass column (2.00 m x 2.0 mm) was packed with 3.8% of SE-30 on Chromosorb W-HP (100-200 mesh). The oven temperature was held 18 min at 185°C, followed by a gradient of 2°/min up to 250°C.

The standard aldolase assay mixture consisted of 0.4 ml Na/K-phosphate buffer (0.1 M, pH=5.4), 3.5 mg Neu5Ac and 1.15 U aldolase. The α-anomer of Neu5Ac was generated in the following mixture: 0.4 ml Na/K-phosphate buffer (0.1 M, pH=5.4), 7.2 mg Neu5Ac(α,2→3)-lactose and 2U sialidase.

Neu5Ac content was measured using a micro-Warren test [12]. For the assay of ManNAc a modified Morgan-Elson colour reaction was used [3].

Optical rotations were measured in a Perkin-Elmer 241 polarimeter. To determine the optical rotation of ManNAc, 54 mg was dissolved in 1 ml water. Measurements were made with time intervals of 15 seconds during 30 min. ManNAc was purchased from Pierce, ICN.K&K and Chemicals Procurement Laboratories Inc.

Results

In situ preparation of α-Neu5Ac

To this purpose Neu5Ac(α,2→3)lactose was incubated with sialidase from Clostridium perfringens. The experiment was performed at pH 5.4 being the optimum pH for the enzyme [10,13-15]. The mutarotation proceeds relatively slowly [8]. In order to quantify the sialidase reaction, ^{1}H-NMR spectra were recorded during Neu5Ac(α,2→3)lactose hydrolysis at 22°C. By integrating the relevant signals, the concentration of all reactants could be calculated every two minutes (fig 2). It is evident that the first reaction product is the α-anomer of Neu5Ac [8]. From the curves of α- and β-Neu5Ac $t_{½}$ for the mutarotation was calculated to be 30 min at pH=5.4 and T=22°C. K_a and K_b were calculated according to [16]. $K_a=3.8\times10^{-4}\ s^{-1}$, $K_b=0.31\times10^{-4}\ s^{-1}$, $K_{eq}=12.16$.

Aldolase reaction

Aldolase was incubated at pH 5.4 with crystalline β-Neu5Ac. No reaction occurred and even after prolonged incubation no ManNAc could be detected. When aldolase was incubated with α-Neu5Ac by including the enzyme in a sialidase/Neu5Ac(α,2→3)lactose mixture at pH 5.4, a rapid conversion to ManNAc took place (at the optimum pH for aldolase (7.2) the rate of the mutarotation is too high to ensure that α-Neu5Ac is the prevailing anomer in solution). The reaction slowed down to 5% of the initial velocity on reaching the Neu5Ac anomeric equilibrium α:β=7.2:92.8.

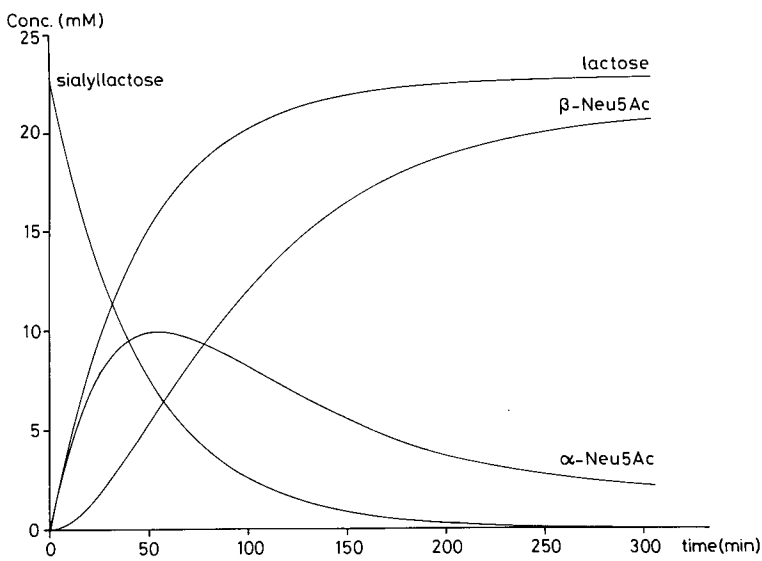

Fig. 2 Time-dependent concentration curves for the reactants in the hydrolysis of Neu5Ac(α,2→3)lactose by sialidase as determined by 360 MHz ^{1}H-NMR spectroscopy. Incubation and NMR conditions are described in Materials and Methods. For Neu5Ac(α,2→3)lactose the peak integral at δ=4.531 ppm corresponding with the anomeric proton signal of galactose was used to calculate its concentration. In the same way for lactose the anomeric signal of galactose at δ=4.452 was used; for α-Neu5Ac H_{3eq} at δ=2.758, H_{3ax} at δ=1.630 and N-Acetyl at δ=2.030 were used; for β-Neu5Ac H_{3eq} at δ=2.227, H_{3ax} at δ=1.801 and N-Acetyl at δ=2.048 were used.

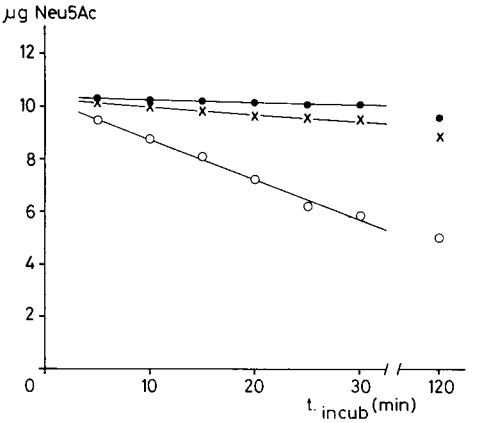

Fig. 3 Reaction of aldolase with Neu5Ac (x——x) and of aldolase plus sialidase with Neu5Ac(α,2→3)lactose (o——o) in 0.1 M Na/K-phosphate buffer pH 5.4 at 37°C. Blanc: Neu5Ac in buffer (•——•).

After storage of β-Neu5Ac for one week in buffer of pH 5.4 the mutarotation equilibrium was reached. Using this anomeric mixture as a substrate, aldolase produced ManNAc at a rate comparable to 5% of the initial velocity of the sialidase/Neu5Ac(α,2→3)lactose reaction. This observation suggests that β-Neu5Ac is not the E-S complex forming substrate.
The aldolase reaction and the mutarotation were also followed discontinuously by analysing aliquots of the reaction mixture either by a colorimetric method or by GLC. The results were in complete agreement with those obtained by ^{1}H-NMR spectroscopy. Fig 3 summarises the results.
From these experiments it is concluded that, at suboptimal conditions with no cleavage of the β-anomer of Neu5Ac, aldolase still possesses the capacity to split the sialidase generated α-anomer of Neu5Ac. In order to get more information on the conformation of the substrate and the necessity of the open chain [17], some kinetic experiments were performed.

Kinetics

The Km for Neu5Ac at pH 7.2 using substrate concentrations of 0.5-5 mM was determined according to [18]. Its value of 1.76 mM is within the range of the published data [3,4,19,20]. Several inhibitors were designed to give more information on the primary substrate. Neu5Ac alditol was prepared as an open chain analog which can neither serve as a substrate nor undergo ringclosure to the semi-aldehyde form. Lacton formation, a common property of N-acetamido-aldonic acids cannot be excluded but a lactone does not have the possibility to form the Schiff's base. If alditol was included in the incubation mixture of Neu5Ac and aldolase at pH 7.2, the enzyme reaction was inhibited competitively with Ki=4.09 mM.

Six ringanalogs lacking the possibility of ringopening, namely the α-methyl, α-ethyl and β-methyl glycosides from Neu5Ac methyl ester and the corresponding compounds with a free carboxyl group were tested. The α-methyl and α-ethyl glycosides inhibited the reaction competitively with Ki=8.10 mM respectively 6.75 mM. The β-methyl glycoside and the three

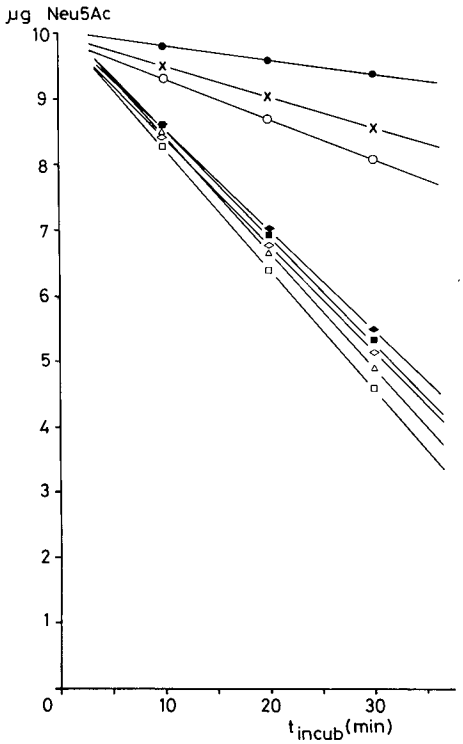

Fig. 4 Reaction of aldolase with 10 mM Neu5Ac (◇——◇) in the presence of equimolar amounts of Neu5Ac alditol (●——●), Neu5Ac α-ethyl glycoside (×——×), Neu5Ac α-methyl glycoside (○——○), Neu5Ac β-methyl glycoside (△——△), Neu5Ac methyl ester α-ethyl glycoside (◆——◆), Neu5Ac methyl ester α-methyl glycoside (■——■) and Neu5Ac methyl ester β-methyl glycoside (□——□).

methyl esters did not influence the reaction. It is concluded that Neu5Ac binds to the enzyme in a conformation that can be inhibited by analogs of the α-pyranose ring. In fig 4 the experiments with one inhibitor concentration are directly compared.

Determination of the configuration of ManNAc

Within the scope of this study it was interesting to determine the anomeric configuration of ManNAc formed in the aldolase reaction. ^{1}H-NMR was not suitable to determine in situ the configuration of this second reaction product since relevant signals coincided with Neu5Ac or were too close to the HOD-signal. An additional problem was the fast mutarotation of ManNAc which interfered with the minimal scantime to obtain useful spectra. Therefore GLC was used to analyze the reaction products in aliquots of the reaction mixture after lyophilization and trimethylsilylation. For the assignment of the α- and β anomers, commercial ManNAc samples were used, all containing the same but unknown anomer. On dissolving this anomer in water, the specific optical rotation shifted within 20 min from -30.4 to +2.9 with $t_{½}$=3 min, k_1=0.088 min^{-1} and k_2=0.13 min^{-1}. After trimethylsilylation the mutarotation equilibrium mixture gave rise to two peaks on GlC. The peak with the longer retention time coincided with that of the derivative of the commercial sample.

The mutarotation was also followed on a Varian MX 390 spectrometer (90 MHz, CW). It was possible to assign the H_1, H_2 and N-Acetyl signals for both anomers. The H_2 and N-Acetyl signals from the anomer in the commercial sample resonated at lower field, whereas the H_1 resonated at higher field than those of the other anomer. In analogy to other sugars [21-23], it is concluded that the commercial samples contain the β-anomer.
Both with NMR and GLC the α/β ratio in the anomeric equilibrium mixture was found to be 1.4. After 30 sec incubation of 9.7×10^{-3} mM Neu5Ac with 0.575 U aldolase at 37°C the ratio had a value of 2.1. This indicated that, after the enzymatic hydrolysis of Neu5Ac, the open chain form of ManNAc preferentially formed the α-anomer after ringclosure followed by a fast mutarotation.

Discussion

We performed several experiments using sialidase to generate the substrate for aldolase. At the optimum pH of sialidase which is 5.4 Neu5Ac is set free in form of its α-anomer. Aldolase possesses still activity at pH 5.4 although its pH optimum is 7.2. α-Glycosides of Neu5Ac inhibit the aldolase action which has led to the suggestion that Neu5Ac binds to the enzyme in a conformation that greatly resembles the conformation of tis α-anomer. This is supported by the observation that the β-anomer does not serve as a substrate and that its β-glycosides do not inhibit the enzymatic action.
Previously, the existence of a covalent bond between C2 of Neu5Ac and the ε-nitrogen atom from a lysine residu in the enzyme was demonstrated [2,4,5]. It was suggested that the formation of an intermediate Schiff's base resulted from the reaction between the carbonyl group of the open chain form with the $\varepsilon\text{-}NH_2$ from lysine. In order to explain our results in terms of such a mechanism the conformation of the open chain form must strongly resemble that of the α-anomer. However, it is also possible that the α-anomer itself binds to the enzyme and undergoes ringopening at the active site. It even cannot be excluded that a nucleophilic attack of the aminogroup on C2 of the intact ring directly results in the formation of the aminocarbinol preceding the Schiff's base.
The observation that the α-ethyl glycoside is a better inhibitor than the α-methyl glycoside might suggest a hydrophobic interaction with the active centre of the enzyme. It could well be that the side chain of the lysine that forms the intermediate covalent C-N bond is involved.

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