Inhibition of N-acetylneuraminic lyase by N-acetyl-4-oxo-D-neuraminic acid

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We show that the 4-oxo analogue of N-acetyl-D-neuraminic acid strongly inhibits N-acetylneuraminic lyase (NeuAc aldolase, EC 4.1.3.3) from Clostridium perfringens (\(K_i = 0.025\) mM) and Escherichia coli (\(K_i = 0.15\) mM). In each case the inhibition was competitive.

N-Acetyl-D-neuraminic acid; N-Acetylneuraminic acid analog; 5-Acetamido-3,5-dideoxy-\(\beta\)-manno-non-2,4-diulosonic acid; 2-Deoxy-2,3-didehydro-N-acetyl-4-oxo-neuraminic acid; Competitive inhibitor

1. INTRODUCTION

NeuAc aldolase is a key enzyme of sialic acid metabolism catalyzing the reversible cleavage of N-acetylneuraminic acid to pyruvate and N-acetylmannosamine [1,2]. The enzyme is, in vivo, predominantly involved in the catabolic pathway [2]. Previous studies indicated that for enzymes involved in sialic acid metabolism C-4 of NeuAc is a crucial area [2-6]. Thus, NeuAc aldolase requires an equatorially oriented hydroxy group at C-4 [2,4,7,8]. Synthetic NeuAc analogues promise to be a useful tool to influence the activity of this aldolase. 4-Deoxy- and 4-epi-NeuAc competitively inhibit the enzyme, both analogues being resistant towards cleavage [7,8]. Based on these results we have synthesized a sialic acid carrying an oxo group at C-4 as inhibitor of NeuAc aldolase. Additionally, the ethyl-\(\alpha\)-glycoside of 4-oxo-NeuAc and the 4-oxo analogue of 2,3-dehydro-NeuAc were studied for aldolase inhibition.

2. MATERIALS AND METHODS

2.1. Materials

All chemicals used were of analytical grade and were purchased from Merck (Darmstadt) or Serva (Heidelberg). Crystalline N-acetylneuraminic acid was prepared in this laboratory. Acetonitrile grade S was obtained from Zinsser (Frankfurt). NeuAc aldolase (5.5 U/mg) from Clostridium perfringens was from Sigma (München), the respective enzyme from Escherichia coli (30 U/mg) and lactate dehydrogenase (500 U/mg) being obtained from Boehringer (Mannheim).

4-Oxo-NeuAc and 2-\(\alpha\)-ethyl-4-oxo-NeuAc were synthesized from the benzyl- and ethyl-\(\alpha\)-glycoside of NeuAc via the diphenyl methyl ester of the 8,9-isopropylidene derivative. Oxidation by CrO\(_3\)/pyridine afforded after removal of the protecting groups the respective 4-oxo analogues [9]. Oxidation of 2,3-dehydro-NeuAc yielded the 4-oxo compound. The detailed syntheses will be described elsewhere.

2.2. Methods

2.2.1. Coupled enzymatic assay

The assay was performed using lactate dehydrogenase (EC 1.1.1.27) as coupling enzyme [8]. NeuAc or 4-oxo-NeuAc were...
tested for cleavage at 5 mM; inhibition was determined at 5 mM NeuAc in the presence of 0.057, 0.114 or 0.228 mM 4-oxo-NeuAc.

2.2.2. NeuAc aldolase assay

This was performed using a modified Morgan-Elson method [8]. The reaction mixture (200 μl) contained 20 μmol KH₂PO₄/K₂HPO₄ buffer, pH 7.2 (C. perfringens aldolase) or pH 7.7 (E. coli aldolase), varying amounts of NeuAc and inhibitor and 20 mU of the respective NeuAc aldolase. The corresponding controls lacked enzyme. The assay was linear with time for 20 min in the absence and presence of 0.0125 mM 4-oxo-NeuAc. Initial rates for the kinetics were determined as described above after 7 min incubation at 37°C.

NeuAc aldolase (C. perfringens) inhibition by 4-oxo-NeuAc was studied as follows. Firstly, NeuAc was varied between 1.25 and 10 mM without inhibitor and in the presence of 0.025 and 0.05 mM inhibitor; secondly, the inhibitor was varied between 0.0125 and 0.125 mM at fixed concentrations of NeuAc (2, 5, 8 mM). Inhibition of NeuAc aldolase (E. coli) was determined by varying the concentration of 4-oxo-NeuAc between 0.1 and 1.1 mM at fixed concentrations of NeuAc (2, 5, 8 mM).

Inhibition of C. perfringens aldolase by 2α-ethyl-4-oxo-NeuAc and 4-oxo-2,3-dehydro-NeuAc was studied as above with inhibitor concentrations adapted to the $K_i$ value.

3. RESULTS

As expected, 4-oxo-NeuAc was no substrate for NeuAc aldolase as determined by the coupled enzymatic assay. However, this analogue proved to be a reversible inhibitor of the enzyme. After dialysis of an enzyme-inhibitor mixture the activity was completely restored. 4-Oxo-NeuAc was stable during incubation at pH 7.2 or 7.7 for 15 min as monitored by analytical HPLC at 200 nm [10]. For determination of the inhibition pattern and $K_i$ value the reaction rate was obtained from the amount of N-acetylmannosamine formed using a modified Morgan-Elson assay [8]. The results agreed with those of studies employing the coupled enzymatic assay (see section 2).

Initial rate data for inhibition of NeuAc aldolase (C. perfringens) by 4-oxo-NeuAc was plotted according to Lineweaver-Burk and Dixon [11]. A competitive inhibition pattern was obtained in both plots. The $K_i$ value of this enzyme was 0.025 mM as obtained from Dixon plots (fig.1), and 0.020 mM as calculated from Lineweaver-Burk plots. The $K_m$ value for NeuAc determined from Hanes plots [11] was 3.75 mM, in agreement with that obtained by using the coupled enzymatic assay [1,8,12]. Dixon plots with E. coli aldolase demonstrated a $K_i$ value of 0.15 mM, the $K_m$ value for NeuAc calculated from Hanes plots being 3.5 mM in accordance with [1]. The $K_i$ value of C. perfringens aldolase for 2α-ethyl-4-oxo-NeuAc determined from Dixon plots (9.5 mM) was high compared to that for 4-oxo-NeuAc. Whereas 2,3 dehydro NeuAc did not inhibit this enzyme, the corresponding 4-oxo analogue showed a $K_i$ value of about 1 mM (Lineweaver-Burk plots). An identical kinetic value was obtained from Dixon plots between 0.188 and 2.25 mM 4-oxo-2,3-dehydro-NeuAc. At higher concentrations of inhibitor the plot changed to a non-linear pattern.
4. DISCUSSION

The action of NeuAc aldolase depends on an unsubstituted hydroxy group at C-4 of sialic acid. Based on the structure of NeuAc only two compounds are known so far to be competitive inhibitors of the enzyme. We have described inhibition by 4-deoxy-NeuAc [4], and this has been confirmed recently [7]. The respective $K_i$ values are 1.25 mM (Morgan-Elson assay [8]; unpublished) and 0.93 mM (coupled enzymatic assay [7]). 4-epi-NeuAc was found to have a $K_i$ value of 2.3 mM as determined by two independent methods [8], whereas a 10-fold lower $K_i$ value was observed with the same analogue synthesized via a different route [7]. The large difference in kinetic data is as yet unexplained.

4-Oxo-NeuAc represents the most potent inhibitor reported as yet, with a $K_i$ value of 0.025 mM for C. perfringens NeuAc aldolase (fig.2). Considering the reaction mechanism proposed for this enzyme [12–14] the oxo group at C-4, instead of that at C-2, may form a Schiff base with lysine at the active site. Alternatively, an enol may arise at C-4 which would interact with the catalytically important histidine. All sialic acid analogues studied in this paper contain an oxo group at C-4 as the prominent structural element. However, the analogues differ in ease of access to the active site of the aldolase, as indicated by the different $K_i$ values. In contrast to unsubstituted 4-oxo-NeuAc, the ethyl glycoside cannot form an open chain. Although this also applies to 4-oxo-2,3-dehydro-NeuAc, in this case the double bond flattens the pyranose ring and may thus induce a more favourable conformation compared to the ethyl $\alpha$-glycoside.

It remains to be seen whether 4-oxo-NeuAc can be employed to block aldolase activity in a mammalian system. It is noteworthy that this analogue is neither a substrate nor an inhibitor for CMP-sialic acid synthase, and that sialidases are not inhibited (unpublished).

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REFERENCES