Methyl α-glycoside of N-thioacetyl-D-neuraminic acid: a potential inhibitor of influenza A virus

A 1H NMR study

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The binding of influenza A virus hemagglutinin to its cell surface receptor, α-linked 5-N-acetylneuraminic acid (sialic acid), was studied in solution. The effect of structural modifications introduced into the N-acetyl group of the sialic acid on the binding was monitored by determining the dissociation constants by proton nuclear magnetic resonance (1H NMR) spectroscopy. Methyl α-glycoside of N-thioacetylneuraminic acid showed high, whereas the corresponding N-methylcarbamoylneuraminic acid exhibited relatively low binding affinity towards the hemagglutinin.

Influenza A virus: Hemagglutinin; α-2-O-Methyl-N-thioacetylneuraminic acid; Binding affinity; 1H NMR

1. INTRODUCTION

The process of adsorption of the influenza virus onto the surface of the host cell, prior to penetration by the viral genome into the cytoplasm, includes the interaction by one of the surface viral glycoproteins, hemagglutinin, with the sialic acid residues bound to cell-surface glycoproteins or glycolipids [1]. The interest in a clarification of the molecular mechanisms by which hemagglutinin and sialic acid interact is twofold. First, it is important to know what chemical groups of hemagglutinin are involved in this interaction and how this interaction can influence the physico-chemical events accompanying the penetration of the influenza virus into the cell; second, if a compound could be found which bound tightly to the region of the hemagglutinin molecule responsible for the interaction with the cell receptor, it would be possible, in theory at least, to block the adsorption of the influenza virus by the cell and thus to prevent infection. This would provide a rational approach to the chemotherapy of influenza infections. The obvious way to treat this problem is to use low molecular weight synthetic derivatives of sialic acid which can be considered to mimic receptor molecules [2,3].

The binding of a variety of sialic acid derivatives to influenza virus and to isolated influenza virus hemagglutinin has been studied using different physico-chemical techniques, namely the inhibition of influenza virus adsorption on erythrocytes [4,5], competition studies using enzyme labelled fetuin [6], 1H NMR spectroscopy [7–9] and X-ray crystallography [10,11]. Sauter et al. [7,9] studied the effect of functional modifications of sialic acid on the binding constant. The removal of those functional groups which, according to the X-ray studies, are involved in the binding, resulted in reduced or abolished affinity. Other modifications had no or litte effect. None of the derivatives they studied showed significant binding affinity.

We report here the 1H NMR analysis of the binding of further, modified sialic acids to hemagglutinin. The main purpose of this work was to find a substance of increased binding activity which would serve as a lead compound for potential inhibitors of influenza virus adsorption by the cell surface receptors.

We screened the following compounds (in brackets is the name used throughout this paper).
- methyl α-glycoside of N-acetylneuraminic acid (acetyl-ligand)
  methyl α-glycoside of N-thioacetylneuraminic acid (thioacetyl-ligand)
- benzyl α-glycoside of N-propanoylneuraminic acid (propanoyl-ligand)
- methyl α-glycoside of N-acryloylneuraminic acid (acryloyl-ligand)

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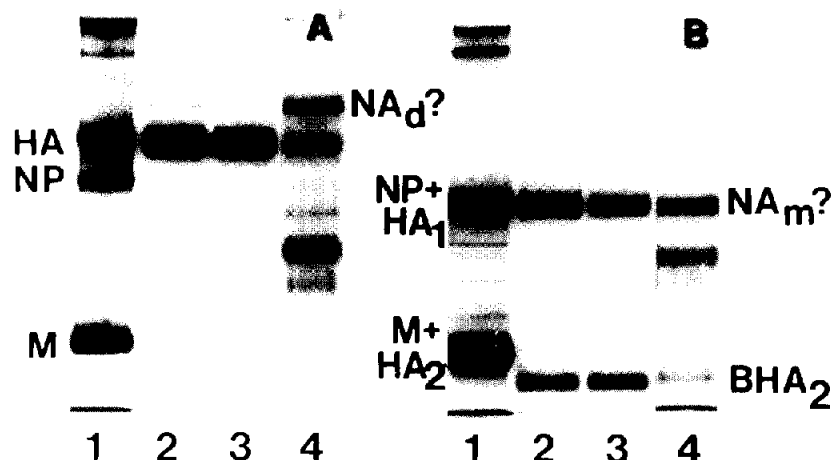
Fig. 1. 10% polyacrylamide gel electrophoresis of influenza virus and viral proteins in unreduced (A, without β-mercaptoethanol) and reduced (B, with mercaptoethanol) conditions. Trace 1, virus; 2, BHA (after sucrose gradient); 3, BHA* (the first peak after column purification); 4, BHA* (the second peak after column purification). Abbreviations: HA (hemagglutinin), NA (neuraminidase), NP (nucleoprotein), M (matrix protein, HA$_1$ (heavy chain of HA), HA$_2$ (light chain of HA), BHA$_2$ (bromelain-released HA$_2$), NA$_d$ (dimer of NA), NA$_m$ (monomer of NA).

- methyl α-glycoside of *N*-thiopropanoylneuraminic acid (thiopropanoyl-ligand)
- methyl α-glycoside of *N*-methylcarbamoylneuraminic acid (methylcarbamoyl-ligand).

2. MATERIALS AND METHODS

2.1. Hemagglutinin purification

Hemagglutinin of serotype H3 was purified from the recombinant X-31 by a modification of the procedure of Brand and Skehel [12]. The hemagglutinin was released from the purified influenza virus with bromelain (Sigma, B-2252) under the following conditions: concentration of the virus, 2 mg/ml; concentration of bromelain, 2 mg/ml (after clarification of the enzyme solution by low-speed centrifugation), incubation at 35°C for 16 h in 0.1 M Tris-HCl buffer (pH 7.2) in the presence of 0.05 M mercaptoethanol and 1 mM EDTA. After ultracentrifugation (Beckman rotor SW-65, 1 h, 10°C, 35,000 rpm) and concentration of the supernatant with a collodium bag concentrator (Schleicher and Schuell), the bromelain-released hemagglutinin (BHA) was isolated from the supernatant by density-gradient centrifugation using Beckmann rotor SW-41 (16 h, 10°C, 40,000 rpm) with a sucrose gradient of 5–20%. After fractionation of the gradient with a fraction collector the collected BHA peak was dialysed against 0.1 M acetate buffer (pH 5.5) and was concentrated using a collodium bag concentrator. Purification of BHA from residual neuraminidase was achieved by using a Sepharose-sulfanilic acid column [13], passing the BHA preparation through the column at pH 5.5 (BHA*). Then BHA* was dialysed against a mixture of 0.02 M D$_2$O-phosphate buffer (pH 7.2), 0.125 M sodium chloride and sodium azide (0.01%). Finally the BHA* was concentrated in a collodium bag concentrator. The concentration of the BHA* was determined by the following methods: Lowry, Bradford, bicinchoninic methods and measurement of the optical density at 280 nm. The purity of the BHA* was checked by polyacrylamide gel electrophoresis (see Fig. 1) and its structure was determined by circular dichroism (CD) measurements at 185–270 nm (α-helix, 29.4%; β-structure, 18.0%; random coil, 52.6%).

2.2. Ligands

Methyl α-glycoside of *N*-acetylneuraminic acid was prepared from per-*O*-acetylated methyl *N*-acetylneuraminate via the corresponding 2-chloro derivative. The propanoyl-ligand was synthesized by reaction of propanoic anhydride with benzyl α-glycoside of neuraminic acid

which in turn was obtained by alkaline de-*N*-acetylation. Reaction of neuraminic acid methyl-α-glycoside with acryloyl chloride afforded the acryloyl-ligand. The detailed synthesis and properties of the thioacetyl and the thiopropanoyl-ligands will be described elsewhere.

2.3. Binding experiments

When a ligand rapidly exchanges between a protein binding site and solvent, the observed chemical shifts (δ_{obs}) and line widths (Δv_{obs}) of the various ligand resonances can differ from the shifts (δ_{free}) and line widths (Δv_{free}) seen in the absence of protein.

The dissociation constant, K_d, can be determined as described by Kronis and Carver [13]. If the fraction of ligand bound to protein is small, K_d is given by either of the following equations:

$$[L]_{TOT} = ([P]_{TOT}/\Delta\delta)\Delta\delta_{B_{app}} - K_d$$

$$[L]_{TOT} = ([P]_{TOT}/\Delta\Delta v)\Delta\Delta v_{B_{app}} - K_d$$

where

$$\Delta\delta = \delta_{obs} - \delta_{free}$$

$$\Delta\Delta v = \Delta v_{obs} - \Delta v_{free}$$

$[P]_{TOT}$ is the total concentration of protein binding sites. $[L]_{TOT}$ is the total ligand concentration. $\Delta\delta_{B_{app}}$ and $\Delta\Delta v_{B_{app}}$ are the apparent chemical shift and line-width changes for the ligand in the bound state. K_d can be determined from the y intercept in plots of $[L]_{TOT}$ versus $1/\Delta\delta$ or $[L]_{TOT}$ vs. $1/\Delta\Delta v$. When $\Delta\Delta v$ values are to be determined, one must be certain that only changes due to the interaction with the protein are measured. Therefore the line width of the trimethylsilyl resonance of TSP (sodium(trimethylsilyl)proprionate) as an internal reference was also monitored and the normalised $\Delta\Delta v$ values were calculated as follows:

$$\Delta\Delta v = (\Delta v_H - \Delta v_{TSP})_{protein} - (\Delta v_H - \Delta v_{TSP})_{free}$$

2.4. NMR spectroscopy

Ligands were prepared for NMR studies by D$_2$O-exchange followed by lyophilization (three times). In order to perform D$_2$O-exchange of BHA the collodium bag containing the protein was placed in deuterated PBS buffer (pH 7.1; 0.15 M sodium chloride, 0.10 M sodium phosphate, sodium azide (0.01%)). Fresh buffer was used three times.
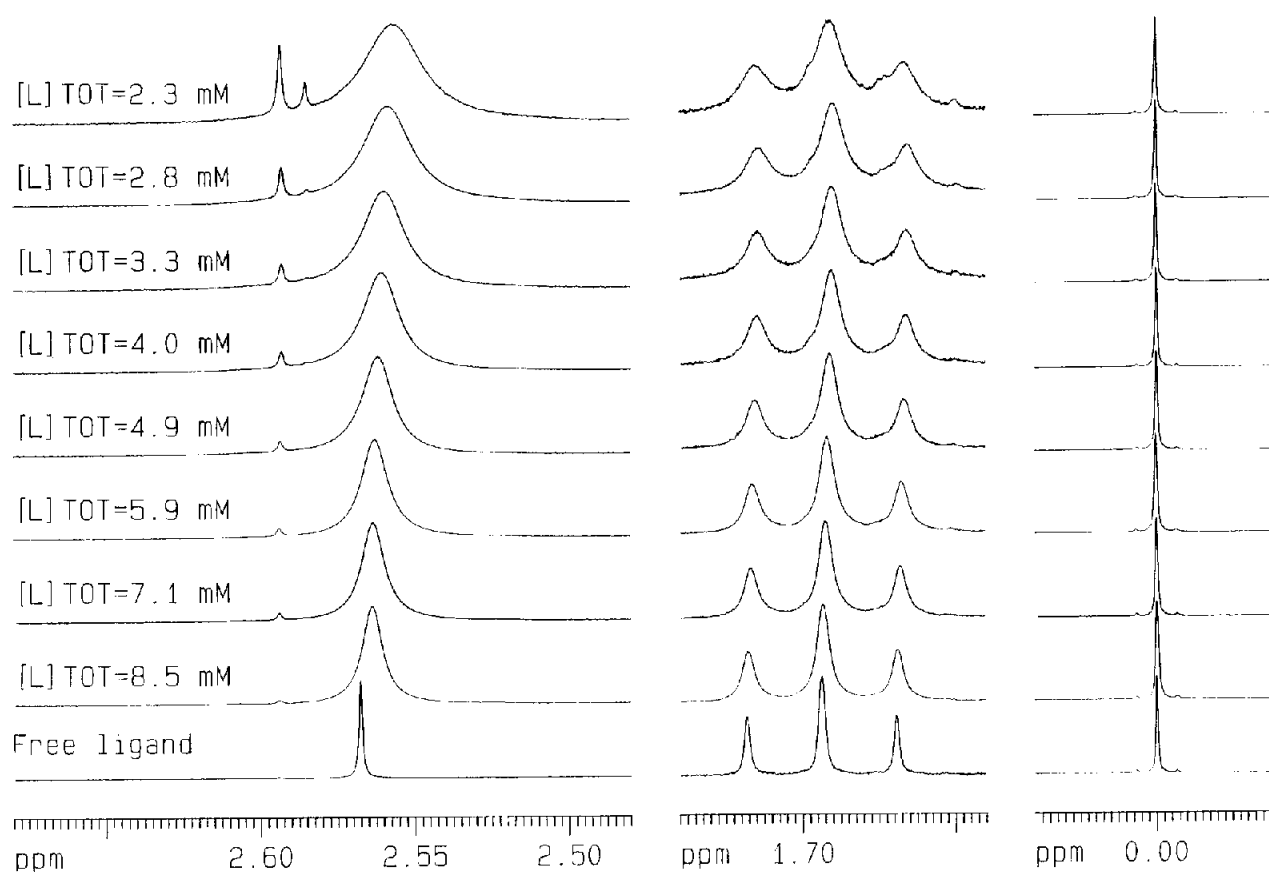
Fig. 2. 500 MHz ^{1}H NMR spectra showing the thioacetyl methyl (singlet, left hand side), the H_{3a} (triplet) resonances of the thioacetyl- ligand and the reference (TSP, right hand side) signal. See section 2 for details.

The measurements were performed in deuterated PBS buffer (see above). Ligand concentration varied between 0.5 mM and 18 mM. BHA concentration was 23 μM or 53 μM. ^{1}H NMR spectra were recorded by a Bruker AMX-500 spectrometer at 298 K. Depending on the ligand concentration, between 32 and 6144 scans were collected. Digital resolution was 0.05 Hz/point. The line widths and chemical shifts were determined using the standard deconvolution programme of Bruker. TSP was used as an internal reference (see above).

3. RESULTS AND DISCUSSION

Fig. 2 shows a typical set of spectra measured on changing the concentration of the ligand (thioacetyl-ligand). The perturbations in chemical shift and line width due to the interaction with hemagglutinin were different for the different resonances. On lowering the concentration of the ligand the perturbations became larger, as a higher fraction of the ligand was bound. For quantitative analysis and the determination of K_d only well-resolved signals could be used which showed large chemical shift and/or line width changes, compared to experimental errors, over a wide range of ligand concentration.

Measurable chemical shift changes were observed only for the methyl group resonances of the actyl, thio-acetyl, thiopropanoyl group and for the acryloyl reso-

Table 1

Ligand	Line analysed	Protein conc. (μM)	K_d (mM)
Acetyl	$H3_{ax}$	23	1.5
	Acetyl	23	1.8
Thioacetyl	$H3_{ax}$	23	0.1
	Thioacetyl	23	0.3
Acryloyl	$H3_{ax}$	51	14
Propanoyl	$H3_{ax}$	23	0.5
	Methylene	23	2.1
	Methyl (propanoyl group)	23	0.7
Thiopropanoyl	$H3_{ax}$	23	1.5
	H5	23	2.1
		13	
	Methyl (thiopropanoyl group)	23	1.8
Methylcarbamoyl	$H3_{ax}$	23	25
	Methyl (methylcarbamoyl group)	23	23

towards the influenza virus hemagglutinin than the natural receptor and any of the compounds so far studied by us and by others. Obviously such compounds possess interesting potential for chemotherapeutic application.

There is evidence that multivalent binding to optimally spaced surface carbohydrate epitopes significantly increases the receptor affinity of influenza virions. Thus, oligosaccharides derived from equine and from human $\alpha2$-macroglobulin, which are similar in structure, have the same low affinity to hemagglutinin. However, equine $\alpha2$-macroglobulin containing multiple optimally spaced oligosaccharides is a high affinity receptor, whereas human $\alpha2$-macroglobulin in which spacing is not optimal, has a low affinity [14]. It will therefore be interesting to see if the binding affinity of the thioacetyl ligand and, thus, its potential as an inhibitor of virus adsorption can be further increased by polymerization under appropriate conditions.

Fig. 3. [L]$_{TOT}$ vs. $1/\Delta\delta$ and [L]$_{TOT}$ vs. $1/\Delta\Delta\nu$ plots for the N- thioacetyl methyl resonance of the thioacetamido-group.

nances. Analysis of chemical shift data indicates the same relative binding potency for the derivatives although the absolute values for the dissociation constants are different.

Fig. 3 shows an example of the plots used for determining the dissociation constants. Table 1 summarizes the K_d values obtained. As seen from the table the analysis of different lines resulted in different K_d values. The results were also different when chemical shifts or line widths were analysed.

Although the K_d values obtained for the same ligand by the analysis of different lines are not the same, the difference between the binding affinity of the different compounds is clear and the data suggest that the thioacetyl-ligand has higher activity towards the hemagglutinin than any of the compounds so far studied by us and by others. The relatively low affinity of the methylcarbamoyl ligand is also unambiguous.

For the first time a sialic acid glycoside monomer has been shown to possess a significantly higher affinity

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