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Proton Nuclear Magnetic Resonance Studies of the Binding of Sialosides to Intact Influenza Virus

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The dissociation constants for binding of sialic acid derivatives to the hemagglutinin on intact influenza virus were determined using nuclear magnetic resonance (NMR) spectroscopy. The dissociation constants determined with whole virus are similar to, but slightly higher than, those determined with BHA (hemagglutinin released from virus by treatment with the protease bromelain; Sauter *et al.*, 1989, *Biochemistry* 28, 8388–8396), indicating that the sialic acid binding site is not significantly altered when hemagglutinin is released from virus. Binding was quantified by observing the concentration-dependent broadening of the sialoside resonances in the presence of X-31 virus or alternatively by observing the effect of the sialoside on the resonances of a competitive "reporter" ligand. The glycosidic substituent attached to the sialic acid makes relatively little difference in the affinity of the sialoside for virus: α(2,6)-sialyllactose (K_D = 2.7 m*M*) binds only slightly more tightly than α(2,3)-sialyllactose (K_D = 3.5 m*M*). However, inversion of the glycosidic center produces a dramatic change in affinity: the dissociation constant for the α-methyl glycoside of sialic acid is 4.2 m*M*, but no binding is observed with the β-methyl glycoside.

INTRODUCTION

The surface of the influenza A virus contains a membrane-bound glycoprotein, hemagglutinin (HA), that binds to cellular receptors containing sialic acid, thereby initiating viral infection (reviewed in Wiley and Skehel, 1987; Paulson, 1985). The primary recognition elements for HA are the sialic acids that are present as the terminal residues on carbohydrates attached to cell-surface glycoproteins and glycolipids. Cells that are treated with neuraminidase to remove sialic acids no longer bind influenza A virus (reviewed in Gottschalk, 1959), but these desialylated cells again bind virus if they are resialyated by sialyltransferases (Paulson *et al.*, 1979) or by addition of sialic-acid-containing glycolipids (Bergelson *et al.*, 1982; Suzuki *et al.*, 1985; Bukrinskaya *et al.*, 1982). The diversity of sialic acids found on cell surfaces (Corfield and Schauer, 1982) and the cooperative, multidentate nature of the binding make it difficult to study virus–cell interactions. Consequently, studies have focused on the binding of simpler, chemically defined sialosides.

Hemagglutination inhibition (HAI) assays have been used to measure the relative affinity of various sialosides to influenza virus (Rogers *et al.*, 1983; Glick *et al.*, 1991; Glick and Knowles, 1991; Spaltenstein and Whitesides, 1991); typically, the concentration of virus is adjusted so that it is four times the minimum amount necessary to agglutinate red blood cells, and the minimum concentration of the sialoside necessary to inhibit hemagglutination under these conditions is then determined. In a closely related technique inhibition of viral adsorption to erythrocytes is monitored by determining the amount of sialidase activity remaining after centrifugation to remove erythrocyte-bound virus (Rogers and Paulson, 1983; Rogers *et al.*, 1983; Pritchett, 1987; Pritchett *et al.*, 1987). Both of these techniques reveal the relative inhibitory potency of different sialosides, but neither provides a direct measurement of the equilibrium-binding constant between the sialoside and HA.

Treatment of influenza virus with the protease bromelain cleaves HA near the viral membrane, releasing a soluble form of hemagglutinin, BHA (Brand and Skehel, 1972). Sauter *et al.* (1989) determined the equilibrium dissociation constants between various sialosides and BHA using NMR. Detailed atomic models of these interactions have been obtained by crystallography (Weis *et al.*, 1988, 1990; Sauter *et al.*, 1992).

To determine whether the equilibrium-binding constants between sialosides and BHA are similar to the binding affinities between sialosides and the HA on whole virus and to further our understanding of the differences in hemagglutination specificities observed for mutant viruses, we extended the NMR binding assay to studies with whole virus. We also describe an

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adaptation of the NMR assay in which the resonances of a competitive "reporter" ligand are monitored as the concentration of the ligand of interest is varied. This competition assay is advantageous in cases in which the resonances of the ligand of interest are not easily monitored, either because they overlap other resonances in the sample or because the concentration of ligand necessary to determine a binding constant accurately are too low to obtain a good signal-to-noise ratio in the spectra (this occurs if the ligand binds relatively tightly to the receptor).

MATERIALS AND METHODS

"NMR buffer"

All experiments were performed in a deuterated buffer containing 0.10 *M* sodium phosphate, 0.15 *M* sodium chloride, 0.1% sodium azide, and 2.5 m*M* sodium (trimethylsilyl)propionate (TSP), 99.96 atom % D_2O, pH = 7.2. pH readings were not corrected for the deuterated solvent.

Virus

All experiments were performed using the recombinant influenza strain X-31 (H3N2). Virus was cultured in embryonated hens' eggs and purified as described (Skehel and Schild, 1971). Virus was prepared for NMR studies by dialyzing into the NMR buffer. The virus concentration in the assay was 2–4 mg/ml (an approximately 20 mg/ml stock solution, upon 100-fold dilution, yielded OD_{280} = 1.022 and OD_{260} = 1.228).

Sialic acids

α(2,6)-Sialyllactose (4), α(2,3)-sialyllactose (5), and disialyllacto-*N*-tetraose (DSL, 6) (Fig. 1) were purchased from Biocarb Chemicals (Lund, Sweden) and used without further purification. α-2-*O*-methyl-*N*-acetylneuraminic acid (Neu5Acα2Me, 1) and β-2-*O*-methyl-*N*-acetylneuraminic acid (Neu5Acβ2Me, 2) were prepared as described previously (Sauter *et al.*, 1989). The synthesis of the α-2-*O*-methyl-4-*O*-phenylpropionyl-*N*-acetylneuraminic acid (Neu4Pp5Acα2Me, 3) will be described elsewhere. Gly(2,2) (7), Gly(4,4), and PEG(5,5) are synthetic, bivalent sialosides provided by Gary Glick and Jeremy Knowles (Glick and Knowles, 1991). A polyacrylamide polymer containing pendant α-sialosides (formed by polymerization of a 10:1 mixture of acrylamide to a sialic acid containing acrylamide derivative) was provided by Andrew Spaltenstein and George Whitesides (Spaltenstein and Whitesides, 1991). The neuraminidase inhibitor 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid (Neu5Ac2en) was purchased from Boehringer-Mannheim Biochemicals and used without further purification.

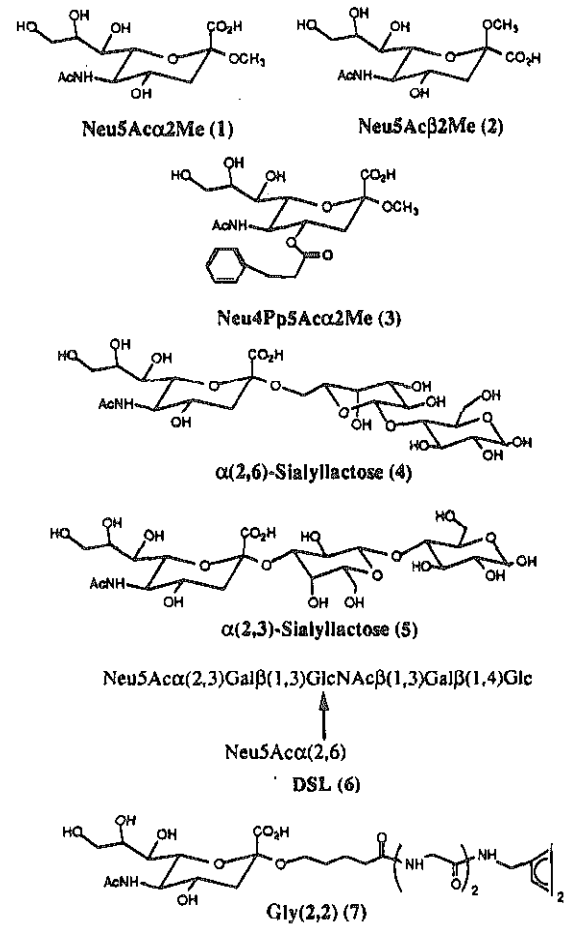

FIG. 1. Structures and abbreviated names for sialosides used in this study.

Stock solutions of the sialic acids were prepared immediately before use by dissolving weighed amounts into a known volume of the NMR buffer. Concentrations of the stock solutions were calculated using the molecular weight of the sialic acids. In the case of 3 the formula weight used in calculating the concentration included 0.5 mole equivalent of H_2O (as estimated from elemental analysis).

During the assay with 5, significant hydrolysis due to neuraminidase was observed, even though the assay solution contained the neuraminidase inhibitor Neu5Ac2en (20 m*M*). At the end of the 2.25 hr necessary for the assay approximately 17% of the sialyllactose was hydrolyzed, as estimated by integration of the α(2,3)-sialyllactose 5-*N*-acetyl methyl resonance relative to the 5-*N*-acetyl methyl resonance of the Neu5Ac formed by hydrolysis. Since accurate integration of the Neu5Ac resonance was not feasible at titration points containing low α(2,3)-sialyllactose concentrations, the

nominal concentrations of α(2,3)-sialyllactose were corrected assuming a linear hydrolysis rate between the first titration point (t = 23 min, 3.8% hydrolyzed) and the final titration point (t = 135 min, 16.6% hydrolyzed).

Binding studies

Using appropriate amounts of the stock solutions described above, two NMR samples, one with virus (V) and one with virus + ligand (VL) were prepared. Both samples contained equal concentrations of virus (approximately 2–4 mg/ml) and the neuraminidase inhibitor Neu5Ac2en (10–20 m*M*). In competition assays both V and VL solutions also contained equal concentrations of the phenylpropionyl derivative **3** (1.0–1.1 m*M*). In addition, the VL solution contained a high concentration (10–20 m*M*) of the sialic acid ligand under investigation. NMR spectra were obtained for these two solutions, and then aliquots of the VL solution were added to the V solution in a series of small steps. NMR spectra were obtained for each of the resulting solutions.

Spectra of the ligands in the absence of virus were also obtained. These reference spectra contained all of the components present in the VL sample, except virus. For each experiment two reference spectra were normally acquired, one containing a ligand concentration near that of the initial VL solution and one containing a ligand concentration 20-fold lower (or, in the case of competition experiments, the second reference spectra contained no ligand).

NMR conditions and analysis of spectra

^{1}H-NMR spectra were acquired on a 500-MHz Bruker AM spectrometer at 297 ± 1 K. We acquired 32K complex data points, with sweep widths ranging from 4000 to 5000 Hz and no recycle delay. Depending on the ligand concentration, between 64 and 1024 scans were collected. Data were transferred to a VAX 8700 and processed with a modified version of the program NMR1 (New Methods Research, Inc.). Free induction decays were directly Fourier transformed (without use of an apodisation function), and the positions and line widths of resonances were determined by modeling singlets with Lorentzian lines and multiplets with a sum of Lorentzians. We observed that the TSP resonance was significantly broadened in the presence of virus. Consequently we used the methyl resonance of the *N*-acetyl group of Neu5Ac2en as our line-broadening standard. (Using the residual HOD resonance as a line-broadening standard gave similar results.)

Analysis of NMR binding studies when two competing ligands are present

Case A: The concentration of a ligand (L1) is varied while the NMR resonances from a second "reporter" ligand (L2), at a fixed concentration, are monitored. Consider two ligands, $L1$ and $L2$, that bind to the same site on a protein (P). The equilibria are described by the following equations where K_D and K_{L2} are dissociation constants and $[P]_f$ is the concentration of free protein binding sites:

$$P + L1 \rightleftarrows P\cdot L1, \quad K_D = [P]_f[L1]/[P\cdot L1], \qquad (1)$$

$$P + L2 \rightleftarrows P\cdot L2, \quad K_{L2} = [P]_f[L2]/[P\cdot L2]. \qquad (2)$$

In the case of fast exchange for the ligand, $L2$, the observed change in line width of a resonance relative to the line width in the absence of protein ($\Delta\Delta\nu_{L2}$) is equal to the fraction of ligand that is bound to the protein (f_{L2}) times the apparent change in line width for the fully bound form ($\Delta\Delta\nu_{Bapp,L2}$), provided that the fraction of $L2$ bound is small. Using $f_{L2} = [P\cdot L2]/[L2]_{tot}$ (where $[L2]_{tot}$ is the total concentration of $L2$), we can write

$$\Delta\Delta\nu_{L2} = \frac{[P\cdot L2]}{[L2]_{tot}}\,\Delta\Delta\nu_{Bapp,L2}. \qquad (3)$$

Noting that $[P]_{tot} = [P\cdot L1] + [P\cdot L2] + [P]_f$ we can write

$$\frac{\Delta\Delta\nu_{L2}}{[P]_{tot}} = \frac{[P\cdot L2]\Delta\Delta\nu_{Bapp,L2}}{[L2]_{tot}([P\cdot L1] + [P\cdot L2] + [P]_f)}. \qquad (4)$$

Substituting for $[P\cdot L1]$ and $[P\cdot L2]$ in Eq. (4) (using Eqs. (1) and (2), and assuming that the fraction of $L1$ and $L2$ bound are small, (i.e., $[L1] \approx [L1]_{tot}$ and $[L2] \approx [L2]_{tot}$) gives

$$\frac{\Delta\Delta\nu_{L2}}{[P]_{tot}} = \frac{\Delta\Delta\nu_{Bapp,L2}}{K_{L2} + [L2]_{tot} + [L1]_{tot}\cdot K_{L2}/K_D}. \qquad (5)$$

Solving for $[L1]_{tot}$ gives

$$[L1]_{tot} = \frac{K_D\Delta\Delta\nu_{Bapp,L2}[P]_{tot}}{K_{L2}\Delta\Delta\nu_{L2}} - K_D\left(1 + \frac{[L2]_{tot}}{K_{L2}}\right). \qquad (6)$$

Thus, if $[L2]_{tot}$ is constant, a plot of $[L1]_{tot}$ versus $1/\Delta\Delta\nu_{L2}$ is a line with a y-intercept of $-K_D(1 + [L2]_{tot}/K_{L2})$. If K_{L2} has been measured independently, then K_D may be readily determined. A similar result is obtained if one looks at chemical shift change rather than line broadening.

Case B: The NMR resonances of a ligand (L1) are monitored as the concentration of the ligand is varied, but this is done in the presence of a fixed concentration of a second ligand (L2). In this case it is the resonances of the ligand $L1$ that are being monitored. Under conditions of fast exchange of $L1$, the observed change in

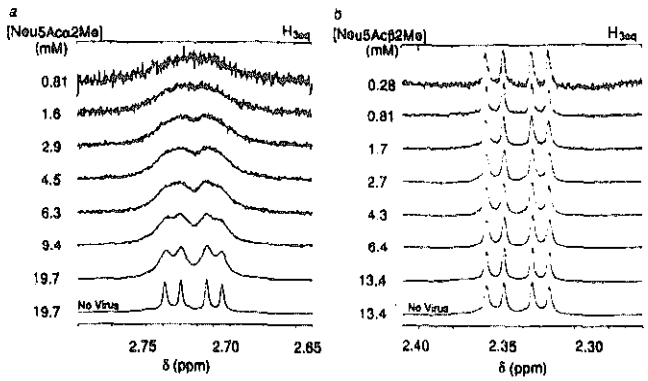

FIG. 2. 500-MHz ^{1}H-NMR spectra of the H3 equatorial (H_{3eq}) resonances of Neu5Acα2Me (a) and Neu5Acβ2Me (b) in the absence (bottom trace) and presence of 2 mg/ml X-31 virus.

line width ($\Delta\Delta\nu_{L1}$) for this resonance is given by the expression

$$\Delta\Delta\nu_{L1} = \frac{[P \cdot L1]}{[L1]_{tot}} \Delta\Delta\nu_{Bapp,L1}. \quad (7)$$

By analogy to the derivation for Case A, it is readily shown that

$$[L1]_{tot} = \frac{\Delta\Delta\nu_{Bapp,L1}[P]_{tot}}{\Delta\Delta\nu_{L1}} - K_D\left(1 + \frac{[L2]_{tot}}{K_{L2}}\right). \quad (8)$$

Thus, if $[L2]_{tot}$ is constant, and $[L1]_{tot}$ and $[L2]_{tot} \gg [P]_{tot}$, a plot of $[L1]_{tot}$ versus $1/\Delta\Delta\nu_{L1}$ is also a line with a y-intercept of $-K_D(1 + [L2]_{tot}/K_{L2})$.

RESULTS

^{1}H-NMR resonances of sialosides broaden in the presence of virus

Our single-ligand (direct) binding experiments are similar to those described previously for BHA (Sauter *et al.*, 1989), except that Neu5Ac2en is added to prevent hydrolysis of the sialosides by neuraminidase present on the virus (Meindl *et al.*, 1971). In the presence of X-31 virus the ^{1}H-NMR resonances of certain sialosides are broadened. Figure 2a illustrates the concentration-dependent broadening of the H_{3eq} proton of **1** in the presence of a fixed concentration of X-31 virus. The other proton resonances of **1** are also broadened. Two observations suggest that the observed line broadening derives from binding to the hemagglutinin and not from binding to neuraminidase or other viral components. First, the β-anomer **2**, which does not bind to BHA (Sauter *et al.*, 1989), exhibits no concentration-dependent line broadening in the presence of virus (Fig. 2b). Second, when virus is treated with Fab fragments derived from antibodies specific to hemagglutinin, added sialosides no longer exhibit line broadening (Eisen, M. B., Skehel, J. J., and Wiley, D. C., unpublished observations).

In addition to broadening, Sauter *et al.* (1989) observed a significant upfield shift in the 5-*N*-acetyl methyl resonances of sialosides upon binding to BHA and found it to be the most useful indicator of ligand binding. If one assumes that the apparent chemical shift change for the fully bound sialoside ($\Delta\delta_{Bapp}$) is the same for binding to virus or binding to BHA, one might anticipate that the relative ratio of broadening ($\Delta\Delta\nu$) to chemical shift change ($\Delta\delta$) would be greater for binding to virus due to its larger size; this indeed is what is observed. For example, in studies with X-31 BHA, conditions that produce a 7.2-Hz increase in line width for the *N*-acetyl methyl resonance of **3** result in a 0.017-ppm upfield shift in this resonance (unpublished results). However, in studies with X-31 virus, conditions that produce a 7.2-Hz increase in line width for the *N*-acetyl methyl resonance of **3** result in only a 0.001-ppm upfield shift in this resonance. Thus, line-broadening measurements are a more sensitive indicator of ligand binding in the experiments described here.

Determination of the dissociation constants by analysis of concentration-dependent broadening of sialoside resonances

For a ligand exchanging rapidly between solution and a protein binding site, and under conditions where the concentration of bound ligand is small relative to the total ligand concentration, the broadening of an NMR resonance is described by the equation (Sauter *et al.*, 1989)

$$[L]_{tot} = \frac{[P]_{tot}}{\Delta\Delta\nu} \Delta\Delta\nu_{Bapp} - K_D, \quad (9)$$

where $[L]_{tot}$ and $[P]_{tot}$ are the total concentrations of ligand and protein binding sites respectively, $\Delta\Delta\nu$ is the observed broadening of the NMR resonance (the difference between the line widths in the presence and absence of protein), $\Delta\Delta\nu_{Bapp}$ is the apparent broadening of the ligand resonance in the fully bound state, and K_D is the dissociation constant. We determined dissociation constants by plots of $[L]_{tot}$ versus $1/\Delta\Delta\nu$. Accurate quantification of the broadening of the H_3 resonances, or other multiplets, was difficult because of the relatively low signal-to-noise ratio and the overlap of the resonances (Fig. 2a). Consequently, we normally analyzed the *N*-acetyl methyl resonance and/or the 2-*O*-methyl resonance of the sialoside.

Figure 3 illustrates the results from the titration of **3** with virus. Analysis of the broadening of the *N*-acetyl

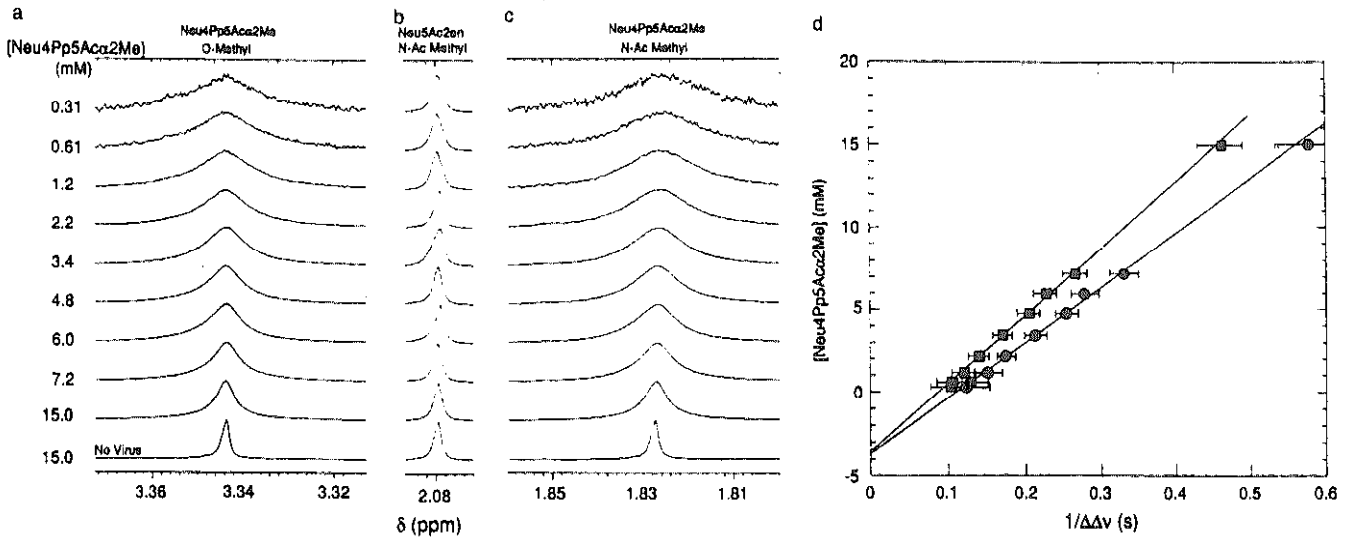

FIG. 3. 500-MHz ^{1}H-NMR spectra of the 2-*O*-methyl (a) and *N*-acetyl-methyl (c) resonances of Neu4Pp5Acα2Me (3). The bottom spectrum was acquired in the absence of virus; all others contained 3 mg/ml X-31 virus. The *N*-acetyl methyl resonance of the neuraminidase inhibitor Neu5Ac2en (b) was used as a line width standard for computing the broadening ($\Delta\Delta\nu$) of the Neu4Pp5Acα2Me resonances in the presence of virus. For example, the broadening of the *N*-acetyl resonance of Neu4Pp5Acα2Me (*N*-Ac) was calculated as $\Delta\Delta\nu = (\Delta\nu_{N\text{-Ac}} - \Delta\nu_{Neu5Ac2en})_{virus} - (\Delta\nu_{N\text{-Ac}} - \Delta\nu_{Neu5Ac2en})_{no\ virus}$. The error in the broadening was defined as the line width in the presence of virus divided by the square root of the signal-to-noise ratio or as the digital resolution, whichever was larger. The data for the *N*-acetyl methyl (●) and the 2-*O*-methyl (■) resonances of Neu4Pp5Acα2Me are shown (d). The lines shown are the results of weighted linear regression analysis (Cleland, 1979). The *y*-intercept is equal to $-K_D$.

methyl and the 2-*O*-methyl resonances according to Eq. (1) gave dissociation constants within experimental error of each other (Fig. 3d; Table 1). The phenylpropionyl derivative **3** is a particularly convenient ligand to analyze since its *N*-acetyl methyl resonance is in an isolated portion of the spectrum (δ = 1.83 ppm), shifted approximately 0.2 ppm upfield from the position normally observed for sialosides. In contrast, the *N*-acetyl methyl resonance of the unsubstituted α-methyl sialoside **1** (δ = 2.04 ppm) occurs quite near the *N*-acetyl methyl resonance of the neuraminidase inhibitor, Neu5Ac2en (δ = 2.08 ppm). Thus, at low concentrations of **1**, the tail of the inhibitor resonance interferes with accurate measurement of the line width of the *N*-acetyl methyl resonance, resulting in a large error in the K_D determination (Table 1). In the case of **1** this is not a major difficulty since analysis of the 2-*O*-methyl group gives a more accurate measurement of K_D. However, in the case of DSL, **6**, and other complex sialosides, accurate determination of K_D is hampered by the lack of resonances that are singlets and that do not overlap resonances of the neuraminidase inhibitor. For example, although DSL contains two sialic acid residues, the *N*-acetyl methyl group resonances lie near the neuraminidase inhibitor *N*-acetyl resonance, and in addition overlap each other. In the case of DSL, we therefore analyzed the H_{3ax} resonances, producing errors in K_D of nearly 50% (Table 1).

Determination of the dissociation constant by analysis of the effect of a titrating ligand on the broadening of a reporter ligand resonance

For ligands whose resonances are difficult to analyze due to overlap or poor signal-to-noise ratio, an alternative method for determining K_D was developed. Instead of monitoring the resonances of the titrating ligand (*L*1), we monitor the resonances of a reporter competitive ligand (*L*2), present at a fixed concentration $[L2]_{tot}$ throughout the titration. A plot of $[L1]_{tot}$ versus $1/\Delta\Delta\nu_{L2}$ (where $\Delta\Delta\nu_{L2}$ is the broadening of the reporter ligand resonance) is a straight line with a *y*-intercept equal to $-K_D(1 + [L2]_{tot}/K_{L2})$ (Materials and Methods, Case A). If the dissociation constant for the reporter ligand (K_{L2}) is known, then the dissociation constant for the titrating ligand (K_D) is readily determined.

Because of the ease with which the resonances of the phenylpropionyl derivative **3** can be analyzed, **3** was selected as the reporter ligand. The concentration of **3** used was approximately 1 m*M*; this concentration is high enough to produce a good signal-to-noise ratio in the resonances being analyzed, but low enough that the resonances are broadened significantly in the absence of the titrating ligand. This concentration is also well below the dissociation constant for **3** (K_{L2}). This not only minimizes the error in the calculated value of K_D due to uncertainty in K_{L2}, it also allows **3** to be

TABLE 1

DISSOCIATION CONSTANTS FOR BINDING OF SIALOSIDES TO X-31 VIRUS

	X-31 Virus				
	Single ligand titrations		Titrations containing a "reporter" ligand[b]		X-31 BHA[a]
Ligand	K_D(m*M*)	Resonance analyzed	K_D(m*M*)	Resonance analyzed	K_D(m*M*)[c]
Neu5Acα2Me (1)	5.6 ± 0.4[d]	2-*O*-methyl	4.0 ± 0.2[d]	5-*N*-Ac methyl of Neu4Pp5Acα2Me	2.8 ± 0.3
	5.2 ± 0.8	5-*N*-Ac methyl	3.8 ± 0.2	5-*N*-Ac methyl of Neu5Acα2Me	
Neu5Acβ2Me (2)	>100	2-*O*-methyl			>200
Neu4Pp5Acα2Me (3)	3.6 ± 0.2	5-*N*-Ac methyl			2.8 ± 0.3[e]
	3.7 ± 0.2	2-*O*-methyl			
2,6-Sialyllactose (4)			2.7 ± 0.1	5-*N*-Ac methyl of Neu4Pp5Acα2Me	2.1 ± 0.3
			2.8 ± 0.2	2-*O*-methyl of Neu4Pp5Acα2Me	
			1.9 ± 0.5	5-*N*-Ac of 2,6-Sialyllactose	
2,3-Sialyllactose (5)			3.5 ± 0.1	2-*O*-methyl of Neu4Pp5Acα2Me	3.2 ± 0.6
DSL (6)	2.8 ± 1.2	H_{3ax} of (2,6)-linked sialic acid			1.2 ± 0.2
	2.8 ± 1.0	H_{3ax} of (2,3)-linked sialic acid			
Gly(2,2) (7)			1.1 ± 0.1	5-*N*-Ac methyl of Neu4Pp5Acα2Me	nd[f]
			1.1 ± 0.1	2-*O*-methyl of Neu4Pp5Acα2Me	

[a] Bromelain-released hemagglutinin.
[b] Neu4Pp5Acα2Me was used as the "reporter" ligand in all competition experiments.
[c] From Sauter *et al.* (1989).
[d] Weighted average from two experiments.
[e] Unpublished results.
[f] Not determined.

readily competed off the viral HA by the titrating ligand. As seen in Figs. 4a and 4d, the resonances of **3** do indeed become sharper as a competitive ligand, in this case **4**, is added. Analysis of either the 5-*N*-acetyl methyl or the 2-*O*-methyl resonance of **3** give similar dissociation constants (Fig. 4e; Table 1). The dissociation constants for binding of **1**, **5**, and **7** were also determined using this procedure (Table 1).

Determination of K_D for a sialoside in the presence of a fixed concentration of a reporter ligand

It is also possible to determine K_D by analyzing the resonances of the titrating ligand ($L1$) rather than the resonances of the reporter ligand ($L2$). A plot of $[L1]_{tot}$ versus $1/\Delta\Delta\nu_{L1}$ again gives a y-intercept equal to $-K_D(1 + [L2]_{tot}/K_{L2})$ (Materials and Methods, Case B). The concentration-dependent broadening of the 5-*N*-acetyl methyl resonance of **4** in the presence of **3** (1.0 m*M*) is shown in Fig. 4c. Although the analysis of this resonance (Fig. 4f) gives a dissociation constant lower than that obtained by analyzing the **3** resonances (Table 1), this discrepancy probably results from the uncertainty in determining the line width of the α(2,6)-sialyllactose *N*-acetyl methyl resonance, due to its proximity to the *N*-acetyl resonance of the neuraminidase inhibitor (Figs. 4b and 4c). In the case of **1**, the dissociation constants obtained by analyzing the titrating ligand resonance and the reporter ligand resonance are within experimental error of each other (Table 1), consistent with our assumption that the ligands are binding competitively.

Viral precipitation is observed with multivalent sialosides

The NMR solutions used in the binding studies are milky due to the high concentration of virus. Addition of

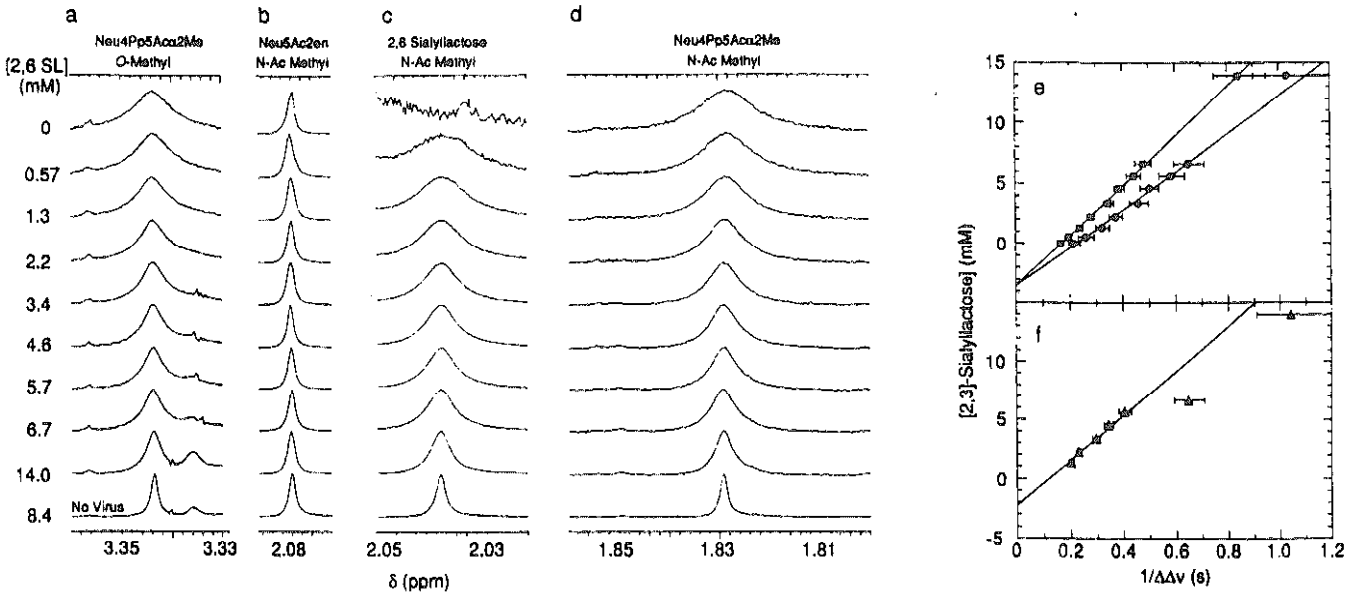

FIG. 4. 500-MHz ^{1}H-NMR spectra for the titration of α(2,6)-sialyllactose (2,6-SL) using Neu4Pp5Acα2Me (3) as a reporter ligand. The 2-*O*-methyl (a) and *N*-acetyl methyl (d) resonances of Neu4Pp5Acα2Me are shown along with the *N*-acetyl methyl resonance of α(2,6)-sialyllactose (c). The Neu5Ac2en *N*-acetyl methyl resonance (b) was used as a line width standard. The concentration of Neu4Pp5Acα2Me is 1.0 m*M*. The bottom trace is in the absence of virus, all others contain 2 mg/ml X-31 virus. The data for the *N*-acetyl methyl (●) and the 2-*O*-methyl (■) resonances of Neu4Pp5Acα2Me are shown (d). The data for the *N*-acetyl methyl resonance of the α(2,6)-sialyllactose are shown (f). In both cases the *y*-intercept is equal to $-K_{2,6\text{-}SL}(1 + [\text{Neu4Pp5Ac}\alpha\text{2Me}]/K_{\text{Neu4Pp5Ac}\alpha\text{2Me}}) = -K_{2,6\text{-}SL} \times 1.27$.

Gly(4,4) (Glick and Knowles, 1991; [Gly(4,4)] = 1.6 m*M*) or a polyacrylamide polymer containing pendant α-sialosides (Spaltenstein and Whitesides, 1991; final concentration of sialic acid groups in solution = 0.05 m*M*) to a solution of X-31 virus (2.0 mg/ml) resulted in a rapid increase in the opacity of the solution. After incubation overnight at 5° a white precipitate formed in the bottom of the solution, leaving a clear supernatant. With the bivalent sialosides PEG(5,5) (1.2 m*M*), DSL (2.7 m*M*), and Gly(2,2) (2.4 m*M*), there were no obvious changes in opacity over the several hours necessary to perform a NMR binding experiment. However, after incubation for several days to several weeks at 5° a precipitate was observed. Similar behavior, precipitation after several days at 5°, was observed with a lower concentration of Gly (4,4) (0.28 m*M*). Precipitation was never observed in NMR solutions containing monovalent sialosides ([Neu5Acα2Me] = 9.4 m*M*, [Neu4Pp5Acα2Me] = 7.2 m*M*, [α(2,6)-sialyllactose] = 6.6 m*M*, [α(2,3)-sialyllactose] = 5.6 m*M*, [Neu5Acβ2Me] = 6.4 m*M*), even after incubation over a month at 5°.

DISCUSSION

Scope and limitations of the NMR binding assay

The use of NMR to determine protein–ligand dissociation constants is a well-established, if not commonly used, technique (Kronis and Carver, 1982, 1985; Perkins *et al.*, 1981; Sauter *et al.*, 1989), and the extension of this technique to the determination of virus–ligand dissociation constants is straightforward. Use of the technique in this way allows dissociation constants to be determined on the physiologically relevant, intact virus. The conventional NMR binding experiment, in which the resonances of a ligand are analyzed as a function of concentration, suffers from several drawbacks that limit its applicability: the resonances to be analyzed must be well resolved from other resonances, the concentration of ligand must be high enough to give a good signal-to-noise ratio for the resonance(s) being analyzed, and the ligand must be exchanging rapidly between the bound and free form. As a result of these constraints only ligands that bind fairly weakly ($K_D \geq 1$ m*M*) and that have relatively low molecular weights can be studied. The use of a competitive reporter ligand, as described in this study, expands the applicability of the NMR binding assay, allowing dissociation constants for more complex and tighter binding ligands to be determined. The reporter ligand may be chosen (or modified) so as to optimize its NMR analysis; it only needs to bind competitively with the ligand(s) of interest and have an easily measured, nonoverlapping resonance. Since the resonances of the ligands under study are not themselves analyzed, the concentration of these ligands may be well below that necessary to obtain a good signal-to-noise ratio.

The NMR binding assay still suffers from the drawback that fairly high concentrations of protein binding sites (>20 μM with BHA) are necessary to produce easily quantifiable changes in the chemical shift or line width of the resonance being analyzed. Since the determination of K_D assumes that the concentration of free ligand is approximately the same as the total ligand concentration, the dissociation constants that may be determined are limited to those significantly above the protein concentration.

Comparison of sialoside affinities to BHA and virus

Our results (Table 1) show that the affinities of simple sialosides for whole virus are similar to, but slightly weaker than, their affinities for BHA (Sauter *et al.*, 1989). As is observed for BHA, the dissociation constants are quite high (>1 m*M*), reflecting the weak interaction between individual sialosides and hemagglutinin. These high dissociation constants are consistent with the observation by Pritchett (1987) that high concentrations of sialosides are necessary to inhibit X-31 adsorption to erythrocytes and with observations that high concentrations of simple sialosides are necessary to inhibit hemagglutination (Toogood *et al.*, 1991).

Although the binding of individual sialosides to virus is weak, the binding of virus to cells is much tighter, presumably due to the multivalent nature of the interaction. Based on the known hemagglutinin inhibition activity of natural, multisialyllated proteins (Chu, 1951) several groups are exploring the importance of multivalency by studying the binding of synthetic multivalent ligands to influenza virus using hemagglutination inhibition assays (Glick *et al.*, 1991; Glick and Knowles, 1991; Spaltenstein and Whitesides, 1991; Roy *et al.*, 1988; Matrosovich *et al.*, 1990; Sabesan *et al.*, 1991). When we attempted to measure the affinity of some of these tighter-binding, multivalent ligands using the NMR binding assay with whole virus we observed aggregation and precipitation of the virus, presumably due to crosslinking of the virus by the multidentate ligands. The rate of this precipitation was qualitatively correlated with the reported relative affinity of the multivalent ligands for HA. Precipitation was never observed with monovalent sialosides.

The *relative* binding affinities of sialosides are similar whether measured with whole virus or BHA. We observe relatively small differences in the affinities of α-sialosides containing different glycosidic substituents. Of particular interest is the relative affinity of 2,6- vs 2,3-sialyllactose. Red blood cells that are resialylated to give exclusively Neu5Acα(2,6)Gal linkages are readily hemagglutinated by X-31 virus, but when resialylated to give Neu5Acα(2,3)Gal linkages no hemagglutination is observed (Rogers *et al.*, 1983; Daniels *et al.*, 1987). In contrast, NMR binding studies with X-31 virus or X-31 BHA show only a small preference for α(2,6)-sialyllactose over α(2,3)-sialyllactose (Table 1). Pritchett (1987) also observed a relatively small difference between (2,6) and (2,3)-sialyllactose binding to X-31 virus. The multivalent nature of the cell–virus interaction may be involved in amplifying the differences observed in the affinities of monovalent sialosides.

As observed with BHA, inversion of the glycosidic position results in a dramatic change in affinity: the dissociation constant for **1** is 4.2 m*M*, but we can detect no binding of **2** to virus. This is consistent with crystal structures of BHA-α-sialoside complexes, in which the sialic acid carboxylate is oriented toward the protein surface making important hydrogen bonds with the protein (Weis *et al.*, 1988).

The similarity between the dissociation constants of simple sialosides for virus and for BHA indicates that the sialic acid binding site is not significantly altered when HA is cleaved with bromelain and released from the viral surface. Thus crystallographic and NMR studies with BHA are accurate reflections of the binding of monomeric sialosides to whole virus. However, further studies with whole virus are necessary to elucidate the mechanism by which the low absolute affinities and low differences in affinities seen with these monovalent sialosides are amplified in polyvalent (e.g., whole-cell) systems.

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