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A Surface Plasmon Resonance Assay for the Binding of Influenza Virus Hemagglutinin to Its Sialic Acid Receptor

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We have developed a sensitive microscale binding assay to study the interaction between influenza hemagglutinin and its cell surface receptor sialic acid using real-time surface plasmon resonance. The glycoprotein fetuin was bound to a carboxymethylated-Dextran sensor surface using *N*-hydroxysuccinimide and *N*-ethyl-*N*′-(dimethylaminopropyl) carbodiimide. Low-pH-induced BHA rosettes bind specifically to the fetuin-derivitized sensor surface, but not to an asialofetuin-derivitized sensor surface. Binding can be inhibited by preincubation of BHA rosettes with millimolar concentrations of inhibitors of the influenza hemagglutinin–sialic acid interaction. The association rate, dissociation rate, and dissociation constant for the multivalent interaction between BHA rosettes and the fetuin-derivitized sensor surface were also measured, allowing us to quantitate the tight binding achieved through the multivalent interaction between BHA rosettes and the fetuin-derivitized sensor surface.

INTRODUCTION

Influenza virus hemagglutinin is responsible for binding virus to target cells by recognizing membrane receptor molecules containing sialic acid (review, Wiley and Skehel, 1987). X-ray crystallographic studies have revealed that the structure of the sialic acid binding site complexed with various sialosides (Wilson *et al.*, 1981; Weis *et al.*, 1988; Sauter *et al.*, 1992; Watowich *et al.*, 1994). The interaction between a soluble form of hemagglutinin and sialic acid has been studied using NMR (Sauter *et al.*, 1989, 1992) and fluorescence polarization (Weinhold and Knowles, 1992) and these studies have found that the dissociation constant is in the millimolar range. Because these assays require milligram quantities of hemagglutinin, it is impractical to make accurate measurements of binding constants of sialic acid to site-directed binding site mutants of the influenza hemagglutinin.

Although the dissociation constant for the binding of hemagglutinin to sialic acid is in the millimolar range, influenza virus is capable of tight binding to the cells it infects because it is capable of binding multiple sialic acid moieties simultaneously. One consequence of this multidentate binding is the observation that influenza virus can hemagglutinate red blood cells (Hirst, 1941). Hemagglutination studies have also been done with influenza virus and modified erythrocytes. The specific modifications have included potassium periodate oxidation (Underwood *et al.*, 1987) and replacement of the native sialic acid with sialic acid in exclusively α2-3 or α2-6 linkages (Carroll *et al.*, 1981) or sialic acid analogues (Higa *et al.*, 1985). Hemagglutination inhibition studies have also been done using sialic acid analogues (Toogood *et al.*, 1991; Glick *et al.*, 1991). These studies are sufficient to distinguish differences in hemagglutination of a factor of 2, but usually only the relative binding between influenza virus and red blood cells has been measured; estimates of the association rate, dissociation rate, and dissociation constant for the multivalent interaction have not been possible.

The protease bromelain cleaves influenza virus hemagglutinin near the viral membrane, releasing a soluble form of hemagglutinin called BHA (Brand and Skehel, 1972). When the pH of a BHA solution is reduced to 5, BHA undergoes a conformational change associated with membrane fusion (Skehel *et al.*, 1982). If the low-pH conformational change is induced in the absence of detergent, the BHA trimers form soluble aggregates called rosettes, containing about 6 to 10 BHA trimers as estimated by sucrose gradient sedimentation and electron microscopy (Skehel *et al.*, 1982). These rosettes are capable of binding sialic acid analogues with approximately the same affinity as BHA, as measured by NMR (Sauter *et al.*, 1992). Rosettes can hemagglutinate red blood cells (Skehel *et al.*, 1982), demonstrating that they are capable of multidentate binding characteristic of the interaction between influenza virus and its target cell.

We have used this multidentate binding in combination

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with the sensitivity of real-time surface plasmon resonance to develop a sensitive microscale assay for the interaction between influenza virus hemagglutinin and its sialic acid receptor. We can demonstrate that the binding observed is dependent on the sialic acid moiety and that preincubation with millimolar concentrations of inhibitors can block binding of rosettes to the fetuin-derivitized sensor surface. We have compared the binding characteristics of wild type X-31 hemagglutinin and the variant V262 to demonstrate the sensitivity and specificity of the binding assay. We have directly measured the association rate, dissociation rate, and dissociation constant of the interaction between rosettes and the fetuin-derivitized sensor surface, with the goal of understanding how tight binding of hemagglutinin and influenza viruses to cell surface receptors is achieved.

MATERIALS AND METHODS

Surface plasmon resonance detection system

All binding experiments were carried out on a BIAcore biosensor system (Pharmacia BioSensor Inc.) using a carboxy-methylated Dextran-coated gold sensor surface (CM-Dextran). The ligand is covalently immobilized to the CM-Dextran surface. A solution containing the protein of interest is then injected over the derivitized sensor surface. The surface plasmon resonance signal (SPR signal), given in terms of response units (RUs), is linearly correlated with the refractive index of the solution in the immediate vicinity of the sensor surface. The SPR signal is measured before and after exposure of the surface by the protein solution. The resulting change in SPR signal is linearly correlated with the amount of protein bound to the ligand (Li *et al.*, 1981; Stenberg *et al.*, 1991).

Immobilization of ligands onto sensor surface

Fetuin and asialofetuin (Sigma) were used without further purification. The CM-Dextran sensor surface was activated using a mixture of *N*-hydroxysuccinimide (NHS) and *N*-ethyl-*N'*-(dimethylaminopropyl) carbodiimide (EDC) as described (Johnsson *et al.*, 1991). Fetuin or asialofetuin was dissolved into a 10 m*M* sodium acetate, pH 4.5, solution at 0.25–0.50 mg/ml and injected over the activated sensor surface for 7–9 min at a flow rate of 5 μl/min. The remaining NHS sites were blocked with a 40-μl 1.0 *M* ethanolamine, pH 8.0. Typically we can immobilize 11,000–15,000 RUs of fetuin or asialofetuin using this procedure.

Preparation of low-pH BHA rosettes

BHA (bromelain-released hemagglutinin) was isolated from the recombinant influenza strains X-31 and V262 as described (Brand and Skehel, 1972). Low-pH-induced BHA rosettes were made by treatment at pH 5.0 as described (Skehel *et al.*, 1982). To remove residual neuraminidase the rosettes were further purified on a 5–25% sucrose gradient in HBS (10 m*M* HEPES, 150 m*M* NaCl, 3.4 m*M* EDTA, pH 7.5) with 0.1 m*M* PMSF in a SW 41 rotor at 34,000 rpm for 16 hr at 10°. Fractions (0.5 ml) were collected and 5 μl from each fraction was analyzed using SDS–PAGE (Laemmli, 1970). Fractions containing low-pH BHA rosettes (usually the bottom four or five fractions) were pooled and dialyzed at least twice into HBS. The concentration of the rosettes in solution was determined by OD_{280}.

Gel filtration chromatography

Sephacryl S-500 HR resin was purchased from Pharmacia and a 30-cm-long × 0.6-cm-wide i.d. column was made according to the manufacturer's instructions. Blue Dextran, thyroglobulin, apoferritin, and β-amylase were purchased from Sigma Chemicals and used without further purification.

Sialic acids

α(2,3)-Sialyllactose and α(2,6)-sialyllactose were purchased from BioCarb Chemicals and used without further purification. α-Methyl sialic acid was synthesized as previously described (Sauter *et al.*, 1989).

Surface plasmon resonance binding experiments

Sucrose-gradient purified BHA rosettes were diluted in HBS to a final concentration of 100–450 μg/ml and exposed to the derivitized sensor surface for 7–9 min at a flow rate of 5 μl/min. During the dissociation phase the sensor surface was exposed to HBS at a flow rate of 5 μl/min. The sensor surface was regenerated using 1 μl 200 m*M* $NaCO_3$, pH 11.5. Between binding experiments the sensor surface was exposed to HBS. For binding experiments with inhibitors the purified rosettes were preincubated with the inhibitor for 3–5 min on ice prior to injection over the sensor surface.

Analysis of kinetics data

The sensorgrams used for direct K_d measurement were analyzed on a NEC 66-MHz 486DX2 using the program BIAEvaluation2 (Pharmacia BioSensor Inc.). The model that fit the dissociation data best was a homogeneous dissociation model. The model that fit the association data best was a homogeneous association model which calculates $K_s = K_a C_n + K_d$ and initial binding rate r_0 by fitting data to the equation $R = r_0/K_s(1 - e^{K_s(t-t_0)})$, where R is the SPR signal, n is the steric interference factor, t_0 is the start time for the association, and C is the molar concentration of the analyte.

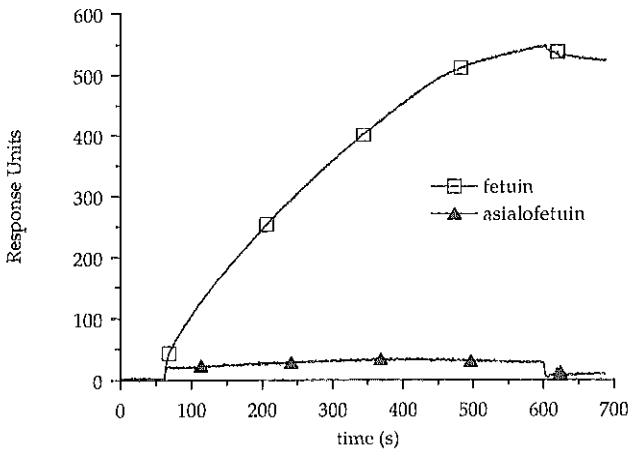

FIG. 1. 45 μl of X-31 BHA rosettes (250 μg/ml) was injected at a flow rate of 5 μl/min over a CM-Dextran sensor surface with a similar amount of fetuin (open squares) and asialofetuin (solid triangles) immobilized (~11,000 RUs).

RESULTS

Low-pH BHA rosettes bind specifically to the fetuin-derivitized CM-Dextran sensor surface

Figure 1 demonstrates that low-pH X-31 BHA rosettes bind to the fetuin-derivitized sensor surface but not to the asialofetuin-derivitized sensor surface. When X-31 BHA rosettes are injected over the fetuin-derivitized sensor surface, there is a rapid rise in SPR signal due to rosette binding to the sensor surface. After the injection is complete, there is a very slow decrease in SPR signal due to bound rosettes dissociating from the sensor surface. On the other hand, when X-31 rosettes are injected over the asialofetuin-derivitized sensor surface, there is an immediate small increase in SPR signal due to the presence of rosettes and sucrose in the solution, but no significant increase during the injection. After the injection, the SPR signal drops to the baseline level. These observations suggest that the binding between the rosettes and the fetuin bound to the sensor surface is due to sialic acids on the fetuin. Similarly, low-pH X-31 BHA rosettes do not bind to an activated and blocked CM-Dextran sensor surface (data not shown), suggesting that the activated and blocked CM-Dextran surface does not contribute significantly to rosette binding to the sensor surface.

Inhibitors of the sialic acid–hemagglutinin interaction block rosette binding to the fetuin-derivitized sensor surface

Figure 2a shows that preincubation of X-31 rosettes with millimolar concentrations of α(2,6)-sialyllactose blocks rosette binding to the fetuin-derivitized sensor surface. In the absence of α(2,6)-sialyllactose there is a rapid rise in SPR signal, just as in Fig. 1. In the presence of α(2,6)-sialyllactose there is a larger increase at the start of the injection due to the presence of α(2,6)-sialyllactose in the solution. During the injection there is a smaller increase in SPR signal, reflecting reduced rosette binding in the presence of inhibitor.

The inhibition of X-31 rosette binding by α(2,6)-sialyllactose was measured at inhibitor concentrations from 0.125 to 4 m*M*, and the results are shown in Fig. 2b. Half-maximal competition occurs at an inhibitor concentration of about 1.2 m*M*. This is similar to the K_d of 2.1 m*M* for the interaction between BHA and α(2,6)-sialyllactose as previously determined by NMR (Sauter *et al.*, 1989). After rosettes have been bound to the sensor surface, subsequent addition of 2 m*M* α(2,6)-sialyllactose did not significantly reduce rosette binding. The inhibition of X-31 rosette binding by α-methyl sialic acid and α(2,3)-sialyllactose was also measured, and the results are summarized in Fig. 3a. We find that the half-maximal inhibition concentration for α-methyl sialic acid is 0.8 m*M* and for α(2,3)-sialyllactose is 2.4 m*M*. Consistent with the NMR and hemagglutination inhibition data, α(2,6)-sialyllactose inhibits X-31 rosette binding more effectively than α(2,3)-sialyllactose. Comparing the difference in half-maximal inhibition concentration between two experiments using the same inhibitor (Fig. 3b), we estimate the error in the measurement of the half-maximal inhibition concentration to be about $\pm$20%. The difference between α(2,3)-sialyllactose inhibition and α(2,6)-sialyllactose inhibition is only slightly larger than the error in the measurements, so care is required in interpreting the data.

The hemagglutinin variant V262 has a single point mutation in HA1 (G135R). V262 virus is able to hemagglutinate periodate-treated red blood cells at periodate concentrations where all other strains tested failed to do so (Underwood *et al.*, 1987). Periodate is thought to remove the glycerol moiety from sialic acid. G135R BHA exhibits a lower affinity for α-methyl sialic acid, α(2,3)-sialyllactose, and α(2,6)-sialyllactose as demonstrated by NMR (Sauter *et al.*, 1992).

We made low-pH-induced G135R BHA rosettes and tested their binding to the fetuin-derivitized sensor surface. G135R rosettes bind to the fetuin-derivitized sensor surface about one-tenth as well as X-31 rosettes, as measured by the concentration of X-31 or G135R rosettes required to generate the same SPR signal on the same fetuin-derivitized sensor surface (data not shown). The inhibition of G135R rosette binding by α(2,6)-sialyllactose and α(2,3)-sialyllactose was measured, and the results are summarized in Fig. 3b. We find that α(2,6)-sialyllactose and α(2,3)-sialyllactose inhibit G135R rosette binding with half-maximal inhibition concentrations of about 2.8 and 3.7 m*M*, respectively, which is greater than the corresponding values for inhibition of X-31 rosette binding. These results are consistent with the NMR results (Table 1). However, when comparing the binding of mutant hemagglutinins, identical methods of rosette preparation may be important in order to generate the same

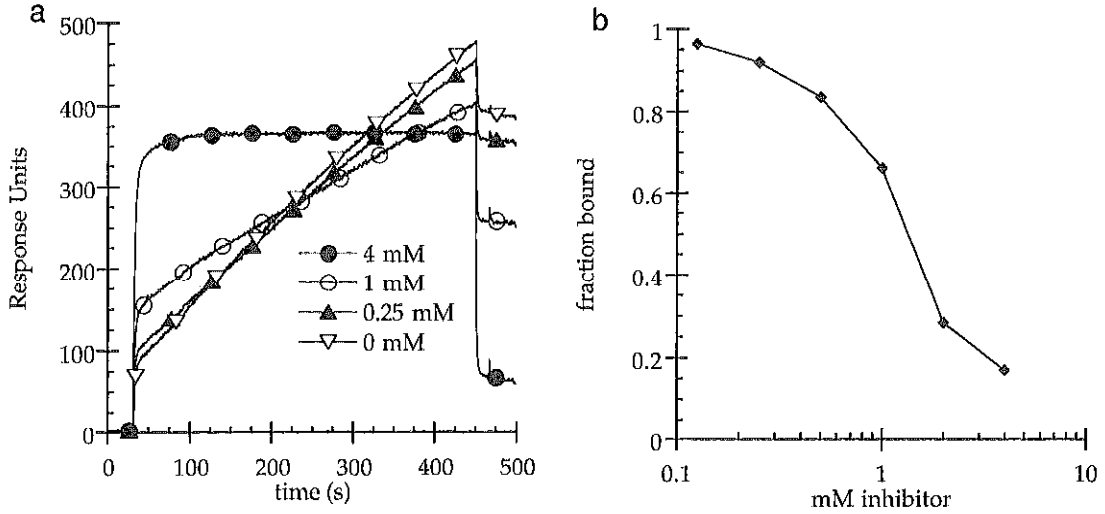

FIG. 2. Inhibition of X-31 BHA rosette binding by α(2,6)-sialyllactose. (a) 35 μl of X-31 BHA rosettes (100 μg/ml) was preincubated for 3–5 min on ice with 4 m*M* (solid circles), 1 m*M* (open circles), 0.25 m*M* (solid triangles), or 0 m*M* (open upside-down triangles) α(2,6)-sialyllactose. Then they were injected at a flow rate of 5 μl/min over a CM-Dextran sensor surface with ~14,000 RUs of fetuin immobilized. The data for 0.125 m*M*, 0.5 m*M*, and 2 m*M* were omitted for clarity. (b) A semilog plot of X-31 BHA rosette binding inhibition by α(2,6)-sialyllactose, given as the fraction of rosettes bound in the presence of α(2,6)-sialyllactose compared to the rosettes bound in the absence of α(2,6)-sialyllactose.

average numbers of hemagglutinin trimers per rosette. Gel filtration chromatography (Sephacryl S-500 HR) indicates that X-31 and G135R rosettes have similar elution volumes and elution profiles (data not shown) and are free of aggregates, suggesting that they have a similar average number of hemagglutinin trimers per rosette.

Determination of the K_d for the interaction between the fetuin-derivitized CM-Dextran sensor surface and X-31 rosettes

To measure the equilibrium dissociation constant for binding of X-31 rosettes to the fetuin-derivitized sensor surface, we obtained binding and dissociation curves at different rosette concentrations. Typical results are shown in Fig. 4. The association and dissociation curves were analyzed as described under Materials and Methods using the program BIAEvaluation2 (Pharmacia Biosensor Inc.). We assumed that there is an average of 8 BHA trimers per rosette, based on previously published estimates of 6 to 10 trimers per rosette (Skehel *et al.*, 1982). Using that estimate, 200 μg/ml of X-31 rosettes corresponds to about a 120 n*M* X-31 rosette solution. To calculate the K_a we need to estimate the steric interference factor, the number of

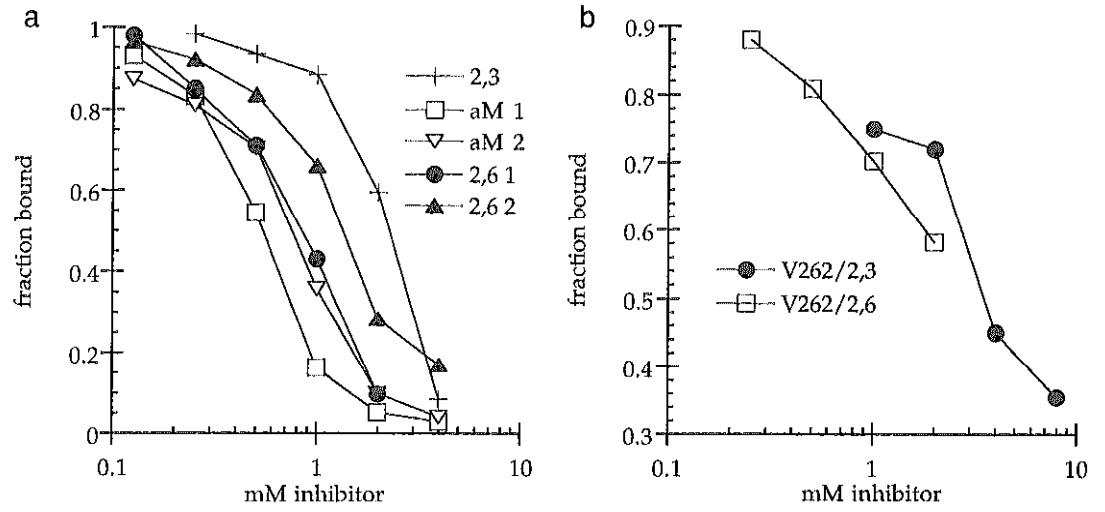

FIG. 3. Inhibition of BHA rosette binding by various sialosides. (a) Comparison of inhibition of X-31 BHA rosette binding by α(2,3)-sialyllactose (crosses), α(2,6)-sialyllactose (solid triangles, solid circles), and α-methyl sialic acid (open squares, open upside-down triangles). Two trials were performed on the α(2,6)-sialyllactose (2,6 1 and 2,6 2) and α-methyl sialic acid (aM 1 and aM 2) samples. (b) Comparison of inhibition of G135R BHA rosette binding by α(2,3)-sialyllactose (solid circles) and α(2,6)-sialyllactose (open squares).

TABLE 1

Inhibition of BHA Rosette Binding by Sialic Acid Analogues

Viral strain	Inhibitor	Half-maximal inhibition concentration (m*M*)[a]	K_d from NMR (m*M*)
X-31	α(2,6)-sialyllactose	1.2 ± 0.2[b]	2.1 ± 0.4[c]
	α(2,3)-sialyllactose	2.4 ± 0.5	3.2 ± 0.6[c]
	α-methyl sialic acid	0.8 ± 0.2[b]	2.8 ± 0.6[c]
V262 (G135R)	α(2,6)-sialyllactose	2.8 ± 0.6	3.7 ± 0.7[d]
	α(2,3)-sialyllactose	3.7 ± 0.7	4.2 ± 0.8[d]

Dissociation constant for binding of X31 BHA rosettes to the fetuin-bound sensor surface

K_a (M^{-1} sec^{-1})[e]	K_d (sec^{-1})	K_d (n*M*)[e]
2×10^3	2×10^{-4}	1×10^2

[a] We estimate an error of ±20% in the measurements.
[b] Based on an average of two measurements.
[c] Sauter *et al.* (1989).
[d] Sauter *et al.* (1992).
[e] Assumes a steric interference factor of 3.

binding sites on the fetuin-bound surface blocked by each binding event. We estimate a steric interference factor of 3 based on the size of the BHA rosette and the expected multivalent nature of the binding interaction. Altering the steric interference factor in the range 2 to 5 will change the association rate constant by less than a factor of 2. The rate constants for association and dissociation K_a and K_d and the equilibrium dissociation constant K_d derived from the analysis are shown in the second part of Table 1. The K_a of 2×10^3 M^{-1} sec^{-1} is lower than expected for a typical protein–ligand interaction, but the K_d of 2×10^{-4} sec^{-1} is extremely slow, resulting in tight binding. X-31 rosettes bind with an equilibrium dissociation constant (K_d) of 1×10^{-7} M to the fetuin-derivitized sensor surface, which is four orders of magnitude tighter than X-31 BHA, X-31 low-pH rosettes, or X-31 virus bound to soluble trisaccharides such as α(2,3)-sialyllactose or α(2,6)-sialyllactose, which are the sialoside constituents of fetuin (Sauter *et al.*, 1989; Hanson *et al.*, 1992). This increased affinity is probably due to multivalent binding between the X-31 rosettes and the fetuin-derivitized sensor surface. Consistent with this view is the observation that X-31 BHA does not measurably bind to the fetuin-derivitized sensor surface (data not shown).

On the other hand, X-31 virus binds tightly and specifically to the fetuin-derivitized sensor surface (data not shown). However, there was substantial loss of binding after removal of the X-31 virus from the sensor surface. This loss of binding could be reduced, but not eliminated, by reducing the amount of X-31 virus bound or reducing the time that X-31 virus was bound to the sensor surface. These results suggest that viral neuraminidase was removing sialic acid from the fetuin, resulting in reduced binding.

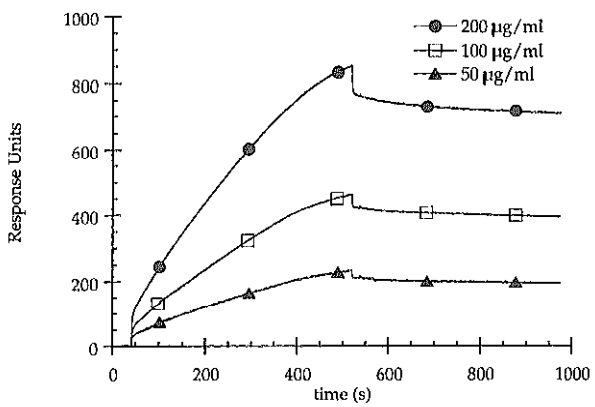

FIG. 4. An overlay of sensorgrams used in K_d determination. 40 μl of X-31 BHA rosettes at concentrations of 200 μg/ml (solid circles), 100 μg/ml (open squares), and 50 μg/ml (solid triangles) was injected over a fetuin-derivitized sensor surface at a flow rate of 5 μl/min. During the 7.5-min dissociation phase which followed, HBS was injected over the sensor surface at a flow rate of 5 μl/min.

DISCUSSION

Perhaps the most important feature of the SPR assay is its small scale. A single injection requires only 80 μl of solution at a rosette concentration of as little as 100 μg/ml, so the total amount of protein required per injection was only 8 μg for X-31 rosettes and 36 μg for G135R rosettes. The small volumes also allow the use of small amounts of the sialic acid derivatives or other synthetic inhibitors for inhibition studies; typically a single inhibition curve can be obtained using less than 0.5 mg of inhibitor, assuming the inhibitor has a millimolar dissociation constant. This assay can be used to screen inhibitors with only a small quantity of inhibitor,

using the half-maximal inhibition concentration as a measure of inhibitory effect. Although the half-maximal inhibition concentrations do not exactly match the dissociation constants obtained using NMR, they are close and usually do match the expected order qualitatively. This similarity in values must be regarded with caution because application of the Cheng–Prusoff correction (Cheng and Prusoff, 1973) or Schild analysis (Arunlakshana and Schild, 1959) may be required to give a more accurate measure how the half-maximal inhibitor concentration relates to the K_i of the inhibitors. Further study would be needed, however, to determine the precise nature of the binding interaction observed on the BIAcore (e.g., number of sites per rosette that must be occupied for rosette binding) in order to calculate an appropriate correction.

The SPR assay can also measure the dissociation constant for the binding of X-31 rosettes to the fetuin-derivitized sensor surface. This is a multivalent interaction similar to the interaction between virus and cell, albeit on a smaller scale. The rosettes bind four orders of magnitude more tightly to the fetuin-derivitized sensor surface than BHA binds to isolated sialosides. The enhancement in binding is due to a very slow off-rate; the dissociation rate constant K_d is $2 \times 10\ \text{sec}^{-4}$, which is very slow for a protein–ligand interaction. This slow off-rate is probably due to the high local concentration of sialic acid binding sites near the sensor surface, so when one sialic acid binding site on a rosette is released from the sensor surface it is very likely that it or another site on the rosette will rebind, thus preventing the rosette from diffusing away.

It is possible to measure the relative binding between variants and site-directed mutants of the influenza hemagglutinin by comparing the amount of rosettes required to get equivalent binding on the same sensor surface, as was done in the case of G135R rosettes. This can even be done when it is not possible to get enough rosettes bound to the sensor surface to get a reliable dissociation constant, as was the case for the poorly binding G135R variant.

Surface plasmon resonance has allowed us to obtain a dissociation constant for a multivalent interaction involving influenza virus hemagglutinin and its sialic acid receptor. The surface plasmon resonance assay can serve as a model system for determining the characteristics of a system where multivalent binding of a weak interaction results in very tight binding, such as the interaction between the influenza virus and the target cell. In conjunction with direct methods such as NMR, this assay can be used to observe the difference in how potential inhibitors bind to single HA trimers compared to how they may block a multivalent interaction, in a manner similar to the way certain bivalent sialosides have been found to inhibit hemagglutination up to 100-fold better than the corresponding monosialosides (Glick *et al.*, 1991). Similarly, this assay may allow one to screen for inhibitors that do not bind soluble trimers well but can inhibit the multivalent interaction that characterizes the binding of influenza virus to its target cell.

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