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Binding of an influenza A virus to a neomembrane measured by surface plasmon resonance

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Abstract—Neomembranes composed of either bovine brain lipid that contains sialoglycolipids or egg yolk lecithin that does not, were formed on an HPA sensor chip and used to study the binding of influenza A virus in real time by surface plasmon resonance. Virus bound only to the bovine brain lipid membrane. This was confirmed by an 84% reduction in virus binding after treatment of the neomembrane with neuraminidase. Binding was temperature dependent, being highest at 30–35 °C and lower at 10 °C. Surprisingly, the rate of complex formation was enhanced, rather than inhibited, by the presence of $1.34–25.2\times10^6$ molecules of free NANA per virus binding site and the rate of dissociation was lower suggesting that the complex was more stable. The free energy of association to form the transition complex was increased by 3 kJ mol^{-1} and there was an almost 10-fold increase in the enthalpy of complex formation in the presence of free NANA. These results show the value of surface plasmon resonance for measuring complex molecular interactions in real time, and provide a model that can be used to study the effectiveness of inhibitors of attachment of influenza virus to its receptor molecules.

1. Introduction

A primary event in many biological processes involved in cell–cell recognition, adhesion, and signalling is the binding of peptides and proteins to specific carbohydrate components of glycolipids or glycoproteins located in the plasma membrane of homo sapien and other animal cells.[1–3] Unfortunately, many bacteria and viruses also use these carbohydrate ligands as the first phase of their invasion strategy.[4,5] Simple monovalent carbohydrate ligands bind to proteins with relatively weak association constants ($K_a = 10^3–10^4$ M^{-1}). Values of this order are rapidly reversible and approach the lower limit for many kinetic measurement techniques such as surface plasmon resonance (SPR). However, the binding strength required to immobilise microbes in biological systems, particularly in sites subject to dynamic flow, is significantly higher and depends upon clusters of ligands at a membrane surface that provide multivalent attachment[6–9] and give the strength of binding required to hold the microbes in place.

A case in point is the specific recognition by the haemagglutinin (HA) spikes on the surface of influenza virus particles of *N*-acetylneuraminic acid (NANA) residues that are component parts of glycoproteins and glycolipids in mammalian cell membranes. Different forms of the virus recognise either α2-3 or α2-6-linked NANA molecules[10,11] and attach themselves tightly to any cell surfaces displaying a high concentration of the appropriate ligands. Influenza virus particles are enveloped and pleomorphic, and between 400 and 1000 HA spikes are inserted evenly into the lipid bilayer. Each spike is a homotrimer with three binding sites for NANA.[12] In addition to the HA spikes, there are about one fifth as many spikes of a virus-encoded neuraminidase on the viral surface that bind to and hydrolyse NANA residues. HA trimers have two different modes

Abbreviations: SA, NANA, *N*-acetylneuraminic acid; NA neuraminidase; HA, haemagglutinin spikes on the surface of influenza A virus; SPR, surface plasmon resonance; HEPES, *N*-[2-hydroxyethylpiperazine-*N*]2-ethanesulfonic acid

Keywords: Influenza A virus; Binding; Surface plasmon resonance; Thermodynamics.

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of interaction with a NANA-bearing cell membranes. Firstly they bind to NANA, which holds the virus close to the surface of the cell membrane, and secondly a multi-step process follows, which results in endocytosis of the virus particle and the fusion of the viral lipid bilayer with that of the vesicle membrane.[13–15]

Many experiments to demonstrate viral binding have been done using liposomes with suitable sialoligands[16] and with erythrocytes[17] and also inhibition of binding.[18] The erythrocyte model involves two complex surfaces, that of the virus and that of the cell, and so it is difficult to study the mechanism in detail. The reaction is normally measured at the end point and any different rates or intermediate steps are not seen. A highly simplified system has been developed to measure the binding of HA trimers (previously liberated from the viral coat by proteolysis) to sialoligands using surface plasmon resonance (SPR).[19] With this technique one can see the whole binding curve and, where differences in the rate of reaction occur, one can analyse the appropriate part of the rate curve to help to interpret what is happening. However, very little work on whole virions has been done. In fact, one of the first uses of SPR to follow the binding of enveloped virions in real time was from this department, and studied the binding of monoclonal antibodies and FAb fragments to covalently immobilised virus.[20]

The main objective of the work described below was to investigate the binding of the human influenza A virus, A/PR/8/34 (H1N1), directly to a neomembrane of bovine brain lipid or an egg yolk lecithin fraction by surface plasmon resonance. In addition, the influence of temperature on the transition state binding of virus alone and in the presence of different concentrations of free NANA was examined.

2. Results and discussion

2.1. Real time binding of virus to neomembranes

Influenza virus binds to NANA residues attached to glycoprotein or glycolipid in membranes. However, it has been claimed[12,21] that influenza virions bind to liposomes of phospholipids alone and undergo some stages of the fusion process. This was not apparent in this study, as virus bound to neomembranes of bovine brain lipid (known to contain a variety of sialoglycolipids) but not to those composed of egg-yolk lecithin (a fraction containing no sialoglycolipid). A typical square wave sensorgram was obtained showing only a bulk change of refractive index change with no binding when virus was passed over egg-yolk lecithin (Fig. 1, curve a). When virus was passed over bovine brain lipid there was a typical binding curve with an association phase (Fig. 1, curve b) This was followed by a minimal desorption phase when buffer alone was passed over the chip surface. Thus under the conditions described, virus interaction was specific and showed no general affinity for membrane lipids. All subsequent experiments were therefore conducted with bovine brain lipid neomembranes using egg-yolk lecithin as a negative control.

The binding of various concentrations of virus from 2.18 to 45.6×10^{-13} M to bovine brain lipid neomembrane is shown in the sensorgram Figure 2. Binding was detectable only at concentrations of virus greater than 1×10^{-13} M. The membrane was saturated at 5.5×10^{-12} M (Fig. 3) and showed a decline in the specific rate of binding at viral concentrations above 5×10^{-13} M. The affinity for the surface was large and a significant residue was left on the sensor chip. A fraction of this material could not be removed by repeated

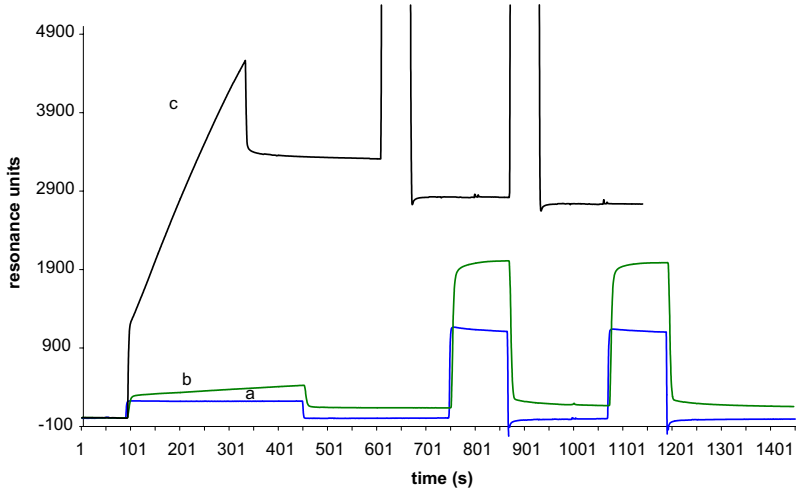

Figure 1. A typical BIAcore sensorgram showing the binding of influenza virus at 28 °C to an HPA sensor chip coated with bovine brain lipid. Curve (a) virus at a concentration of 1.45×10^{-12} M passed over a negative control surface of egg-yolk lecithin and failed to show any binding, curve (b) binding of virus alone at a concentration of 1.45×10^{-12} M, curve (c) binding of the same concentration of virus in the presence of added free NANA (22.6 mM). Both binding curves show an association phase of the virus followed by a dissociation phase when buffer alone was passed over the chip surface. The two large peaks to the right are pulses of 50 mM NaOH used to remove the residual virus and regenerate the sialoglycolipid surface. This was more successful when NANA was present during association of the virus. The square wave shape of curve a is due to a bulk change in concentration (and therefore a change in refractive index). There is no binding gradient.

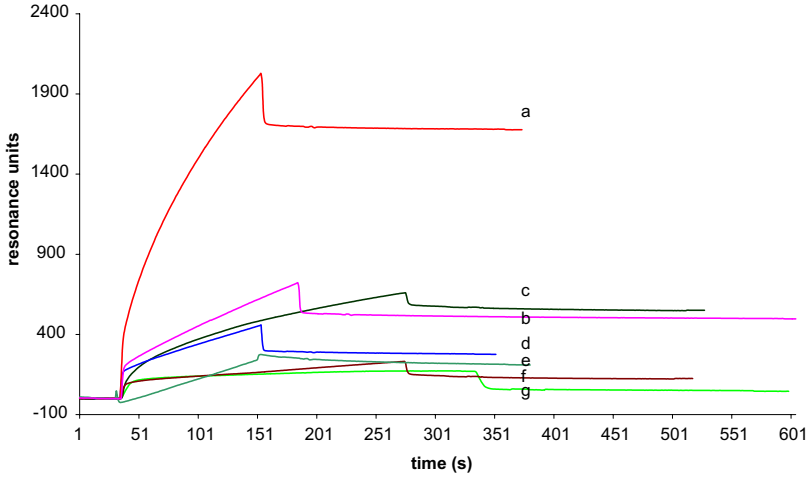

Figure 2. A sensorgram of different concentrations of virus binding to brain lipid at 28 °C. Curve $a = 4.56 \times 10^{-12}$ M, $b = 2.78 \times 10^{-12}$ M, $c = 2.29 \times 10^{-12}$ M, $d = 5.01 \times 10^{-13}$ M, $e = 3.77 \times 10^{-13}$ M, $f = 2.5 \times 10^{-13}$ M, $g = 2.18 \times 10^{-13}$ M.

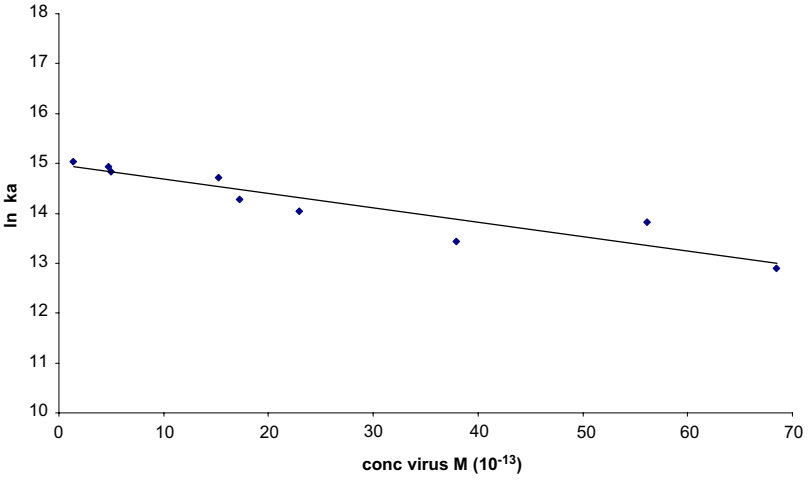

Figure 3. A plot showing the association rate of virus with concentration to a brain lipid neomembrane on a sensor chip at 28 °C. The rate of binding fell as increased titres of neuraminidase (from the higher concentration of virus) influenced the reaction. Kinetic measurements were made at concentrations between 1 and 6×10^{-13} M virus.

exposure to NaOH. Attempts to regenerate the chip with 100 mM HCl resulted in surface degeneration and fragments of the neomembrane were detached and lost. Acid treatment may have resulted in exposure of the hydrophobic membrane fusion N-terminus of the HA2 membrane anchor that is known to unfold in an irreversible new conformation when exposed to an acid pH.[22] Washing the chip surface with buffer at pH 7.4 for 1–2 h restored a stable surface that could be used for further measurements. A surface that had degraded to an extent that it failed to give reproducible results could be replaced by a new surface. New batches of lipid were fused to the sensor chip at 30 °C. Moreover, this allowed a change of lipid (e.g., from egg yolk to brain lipid) and in this way the same chip was used for multiple measurements over several weeks.

A number of experiments were done to standardize the binding curves. The absence of binding to egg-yolk lecithin allowed this mixture of phospholipids to be used as a negative control on track 1 of the sensor chip. Any slight variations in the four traces due to artifacts produced by the machine and by bulk changes in refractive index were eliminated by deducting the values obtained for track 1 from the experimental tracks 2–4. The flow rate was investigated to see whether mass transfer was a problem. At flow rates above 25 μL min^{-1} there was no evidence of mass transfer problems. To study the effect of neuraminidase on the binding of HA to sialoglycolipid sensorgrams were run for different times and with different concentrations of virus. It was observed that on long runs (>250 s) and particularly at higher concentrations of virus ($>8 \times 10^{-12}$ M) there was an inflection in the sensorgram followed by a second part of the curve with a higher rate of association (not shown). For the kinetic study (see later) conditions were chosen to remove the secondary reactions.

2.2. Effects of bacterial NA on virus binding to neomembranes

Neomembranes not previously exposed to virus were comparatively stable for 2–3 days and gave good binding

of virus. However after exposure to virus, neomembranes degenerated overnight, even after regeneration with alkali. It was not possible to totally remove the virus with alkali (Fig. 1). Repeated injection of virus gave similar sensorgrams with a slightly reduced slope on each subsequent injection, consistent with residual virus on the surface blocking some of the sites and viral NA, on the virus surface, removing some of the NANA from the neomembrane. When externally derived NA (from *Clostridium perfringens*) was added to the neomembrane there was a concentration-related loss of signal and consequently most the sensorgrams had a negative slope. Four concentrations of 0.02, 0.033, 0.05 and 0.140 mM of bacterial NA were passed over the surface. Each injection was followed by an injection of free NANA to limit the destruction of the neomembrane and hopefully to complete the desorption of the enzyme. After each injection of NA the base line was progressively lowered due to the action of the enzyme (data not shown).

Table 1. Influence of temperature on the hydrolysis of sialic acid groups from the brain lipid neomembrane

Temperature (°C)	Neuraminidase concentration (mM)	Slope (rate of binding dRU/dt)
10	0.02	0.10
	0.33	0.06
	0.05	0.05
	0.14	−0.04
20	0.02	0.07
	0.33	−0.03
	0.05	−0.04
	0.14	−0.08
28	0.02	−0.01
	0.33	−0.03
	0.05	−0.06
	0.14	−0.13

Neuraminidase was a Sigma Type V preparation from *Clostridium perfringens*.

The binding of bacterial NA to the neomembrane was detectable at low temperatures (10 °C) where the enzyme activity was low, but at 20 °C and above the slopes of the curves were all negative (as expected) showing degradation of binding sites (Table 1). Injection of virus after exposure of the neomembrane to NA at 28 °C caused a significant reduction (84%) in the slope of the sensorgram when compared to a sensorgram of a track not exposed to neuraminidase (Fig. 4). These data confirm that the influenza virus was binding to sialoglycolipid specifically on the neomembrane surface.

2.3. Free NANA stimulated virus binding to neomembranes

Attempts were made to inhibit the binding of the virus to the neomembrane by pre-incubation of virus with free NANA. It is known that 30–50 mM NANA will totally inhibit viral absorption to derivatized erythrocytes,[7] and therefore various concentrations up to 22.6 mM were tried with low concentrations of virus. It was expected that there would be some inhibition of virus binding because previous experience of the authors with other systems has shown that the presence of free ligand mixed with the analyte competes with the bound ligand. However, the sensorgrams showed a large increase in binding (Fig. 1, curve c) compared with the results obtained when the virus had no prior exposure to NANA (Fig. 1, curve b). Moreover, as the ratio of the concentration of virus to NANA increased, there was a corresponding fall in the rate of binding, at 28 °C (Fig. 5). There are at least 4:1 HA-trimers to NA units on the viral surface. Therefore, the NA controlled processes will be slower than the HA controlled ones, particularly in the initial phase of the reaction curve and this idea was used in the kinetic analysis. It was expected that the large number of free NANA molecules (a minimum of 4.5×10^5 molecules per HA trimer) would inhibit neuraminidase and therefore reduce release of the virus and

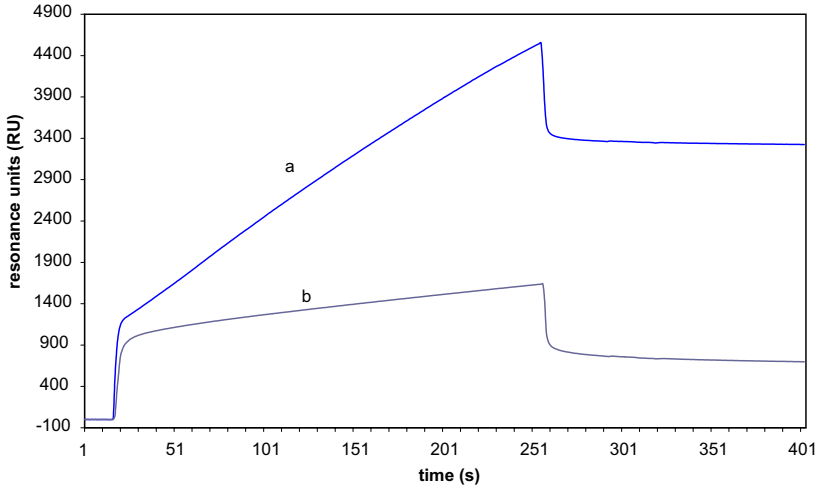

Figure 4. Virus binding at 28 °C was decreased by treatment of the neomembrane with bacterial neuraminidase (*Clostridium perfringens*). Curve (a): virus binding to a neomembrane untreated with neuraminidase, curve (b): virus binding to a parallel track on the same chip where the neomembrane had been treated three times with neuraminidase prior to exposure to virus. Free NANA was used to remove residual enzyme and virus binding was carried out in the presence of NANA. The reduction in binding was 84%.

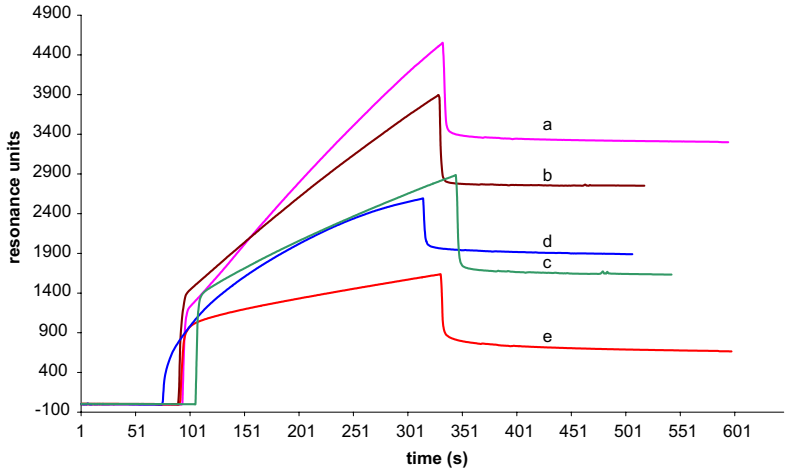

Figure 5. A sensorgram showing the binding at 28 °C of different concentrations of virus in the presence of free NANA (11.3 mM). Binding was more rapid when the ratio of virus to NANA was low. Curve $a = 2.78 \times 10^{-13}$ M, $b = 3.77 \times 10^{-13}$ M, $c = 4.56 \times 10^{-13}$ M, $d = 5.01 \times 10^{-13}$ M, $e = 9.13 \times 10^{-13}$ M.

the degradation of binding sites on the surface. The association constant between sialoligands and the HA is high compared with that for free NANA and therefore high concentrations of free NANA are required to compete with the sialoglycolipid ligand on the neomembrane surface. Recent experiments on a series of *N*-haloacetyl substituted neuraminic acids that were shown to be inhibitors of neuraminidase support this idea and showed similar and often greater increases in binding of virus compared with that observed in the presence of free *N*-acetyl neuraminic acid.[23]

2.4. Effects of temperature on virus binding to neomembranes

Carefully chosen conditions were used for the kinetic measurements. Firstly, the number of virus particles (and hence the viral neuraminidase concentration) was kept at a minimum level compatible with obtaining a binding signal. Secondly, the time of exposure of virus to the surface was limited to a time when saturation was almost complete (150 s for virus alone and 250 s when NANA was present), again to limit the accumulation and action of neuraminidase. Sensorgram curves (Fig. 5) produced using the BIAcore were analysed using the BIAevaluation software that enables one to calculate the k_{on} rate constant for viral binding to the surface. Separate analysis of the desorption curves gave a figure for the k_{off} rate constant. A detailed description of the method of analysis has been published.[24,25] Under the conditions used the analyte passing over the surface is in excess and any potential influence of neuraminidase is minimized allowing the reaction to be treated as pseudo-first order.

We explored the effect of temperature on the binding of virus particles to the neomembrane, and on the virus

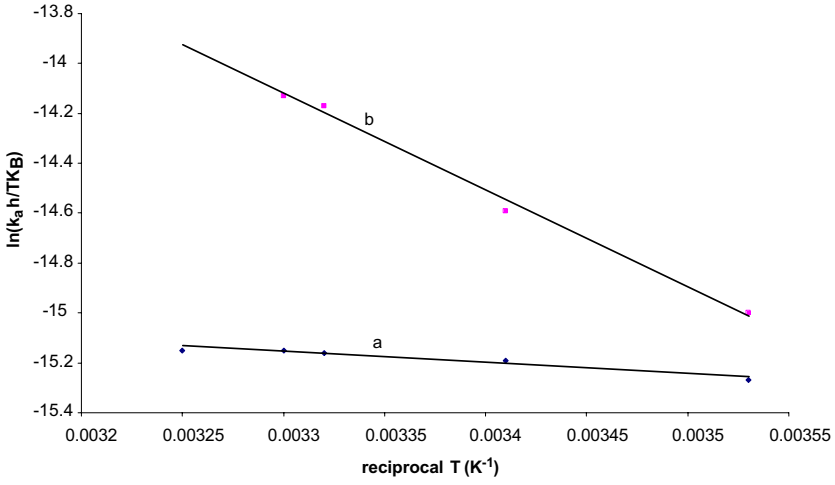

Figure 6. An Eyring plot of the association rate constants against the reciprocal of the absolute temperature showing the formation of the transition state complex between virus and sialoglycolipid on the surface of the HPA chip. Curve (a) virus alone and curve (b) virus with added free NANA. The rate of association increases with temperature as predicted from collision theory. The standard deviation on all points was low (±0.016 for virus alone and ±0.021 for virus + NANA). Note: the Boltzmann constant is symbolized by k_B.

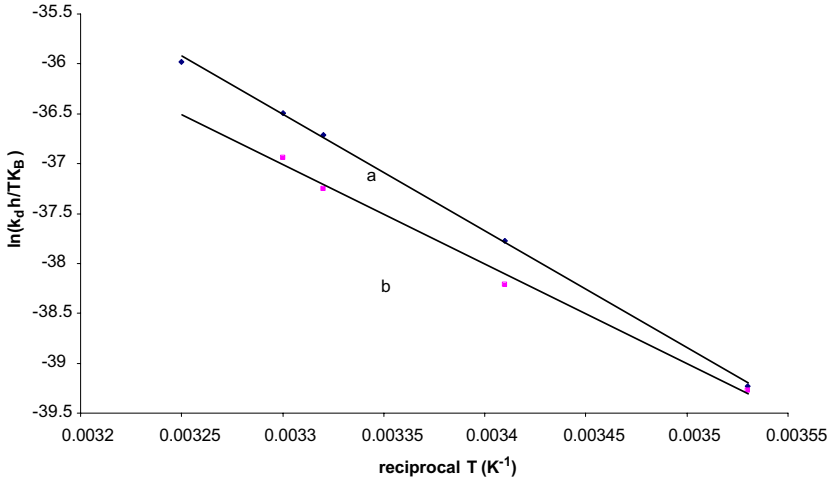

Figure 7. An Eyring plot of the dissociation rate constants against the reciprocal of the absolute temperature showing the dissociation of the transition complex between virus and sialoglycolipid. Curve (a) virus alone and curve (b) virus with added free NANA. The rate of dissociation increases rapidly with increased temperature as the complex becomes less stable. The standard deviation for virus alone was 0.081 and ±0.15 for virus + NANA, higher than found for the association phase.

binding in the presence of NANA. Binding was highest at 30–35 °C and was lowest at 10 °C. Similar results were obtained when NANA was present but in this case the magnitude of the effect was more dramatic because there was a two-fold increase in the specific rate of association of virus. These results are illustrated in the Eyring theory plots (ln $kh/k_{\aleph}T$ versus $1/T$) (Figs. 6 and 7). The calculated values for the change of free energy, enthalpy and entropy of the transition state binding are shown in Table 2 with and without NANA. The free energy for both formation of the transition state complex and dissociation of the complex are slightly higher for the virus in the presence of free NANA than for the free virus alone. The higher values for the free energy of formation of the transition complex arise because the enthalpy of association is almost 10-fold higher than that for virus alone. Although the enthalpy of dissociation of the complex is lower when NANA is present this is compensated for by a favourable entropy term. Calculated values for the change of free energy, enthalpy and entropy at equilibrium are presented in lower part of Table 2. The free energy values are almost the same but the enthalpic change on binding of virus alone is much larger than when NANA was present. There is a small favourable entropic change in the virus/NANA reaction whereas with virus alone the entropic change is unfavourable. The equilibrium values are those for a polyvalent interaction of the virus with the neomembrane sialoglycolipid and not those of the standard state ΔG^{o}. Therefore, the average free energy of interaction, $\Delta G_{\mathrm{av}}^{\mathrm{poly}}$ between a single ligand and a single receptor moiety in the polyvalent interaction is equal to $\Delta G_{N}^{\mathrm{poly}}/N$. A monovalent ligand–receptor interaction occurs with a free energy change of ΔG^{mono}. Also, N monovalent independent receptors interact with N monovalent independent ligands with a free energy change of $N\Delta G^{\mathrm{mono}}$.

Thus, $\Delta G_{\mathrm{av}}^{\mathrm{poly}} = \Delta G_{N}^{\mathrm{poly}}/N$ and since $\Delta G = -RT\ln K$.

Then $K_{N}^{\mathrm{poly}} = (K_{\mathrm{av}}^{\mathrm{poly}})^{N}$. For more details of these calculations see the work of Mammen et al.[8]

Table 2. Kinetic data and calculated thermodynamic parameters from transition theory and Eyring plots

	Virus alone	Virus + NANA
Temperature range (°C)	10–35	10–30
K_a range ($10^6\,M^{-1}\,s^{-1}$)	1.43–1.71	1.83–4.69
K_d range ($10^{-4}\,s^{-1}$)	0.58–16.45	0.42–5.16
Association ΔG^{*} (kJ mol^{-1})	54.4	57.5
Dissociation ΔG^{*} (kJ mol^{-1})	−111.3	−113.9
Association ΔH^{*} (kJ mol^{-1})	3.8	32.2
Dissociation ΔH^{*} (kJ mol^{-1})	98.0	83.2
Association $T\Delta S^{*}$ at 298 (kJ mol^{-1})	−50.6	−25.4
Dissociation $T\Delta S^{*}$ at 298 (kJ mol^{-1})	−13.3	−30.7
Equilibrium[a] values in kJ mol^{-1} (except for ΔS)		
$\Delta G_{\mathrm{equil}}$	−56.9	−56.4
$\Delta H_{\mathrm{equil}}$	−94.2	−51
$T\Delta S_{\mathrm{equil}}$	37.3	−5.3
$\Delta S_{\mathrm{equil}}$ J K^{-1} mol^{-1}	125	−18

[a] Note: the equilibrium values describe a polyvalent interaction (see Discussion).

As we do not know the value of N it is not possible to calculate an accurate figure for the equilibrium constants K_A and the K_D for a single ligand/analyte interaction. Calculations (not shown) that are based on the area occupied by HA trimers on the virus surface and the distance between them (not allowing for any surface deformation) suggest that there may be 7–9 spikes that are able to bind, assuming no steric hindrance. The calculations also show that there is an advantage to a virus that produces longer spikes. This increases the number of anchor points on a surface. A further advantage accrues for pleomorphic forms of the virion that are able to orientate more binding spikes along the surface of a membrane.

3. Conclusions

Using SPR we have confirmed that influenza virus A/PR/8/34 binds specifically to a neomembrane (bovine brain lipid) that contains NANA. A reduction in virus binding following exposure of the neomembrane to NA was consistent with this observation. Binding followed Eyring theory that relates temperature to specific rate, up to a temperature of 35 °C. In the presence of free NANA the rate of binding per second increased 10-fold, an observation that, in relation to the proportion of NANA to virus, was counterintuitive. The equilibrium enthalpy of the interactions of virus alone was almost double that when NANA was present and the easier binding of the virus in the presence of NANA was favoured by an entropic contribution, unlike the binding of virus alone. The inhibition of virus NA activity by excess free NANA contributes to the enhanced stability. Further investigations using pure lipid membranes and improved NA inhibitors are required to probe the equilibrium thermodynamic parameters in more detail.

4. Experimental

4.1. Preparation of the neomembrane

Bovine brain lipid (Type VII) or an egg yolk lecithin fraction (Type XV E) (10–15 mg; Sigma) was suspended in 10 mL buffer (0.01 M HEPES, 0.15 M NaCl pH 7.4) and homogenized thoroughly (×3) at 35 °C. These suspensions were injected (325 μL) over one track at a time of an HPA sensor chip that has a hydrophobic surface (BIAcore), at 30 °C in a BIAcore 2000 instrument at a flow rate of 5 μL/min, and allowed to fuse. The procedure was then repeated. Tracks were washed between injections with a fast flow of buffer to remove excess layers of lipid and with 50 mM NaOH (2×15 μL) to produce a stable neomembrane. Experiments involving the preparation of unilamellar liposomes by sonication and subsequent application of the liposome suspension to the HPA chip surface produced the same sensorgrams when surfaces were exposed to virus. Therefore, preparation of liposomes gave no advantage over homogenized sialoglycolipid. The same HEPES buffer solution was used through all experiments.

4.2. Preparation of virus and its binding to neomembranes

Human influenza A virus strain A/PR/8/34 (PR8; H1N1) was prepared by standard procedure from allantoic fluid of fertilized chickens' eggs that had been inoculated with virus, and purified by centrifugation on successive velocity and density gradients of sucrose. Virus was assayed by haemagglutination titration using standard methods, and analysed for total protein and by quantitative polyacrylamide gel electrophoresis. The concentration of physical virus particles was calculated on the basis of an average figure of 2883 molecules of matrix (M1) protein per virus particle.[26–28] The molecular weight of the virus was accepted as 2.5×10^8.[29] Virus was diluted in HEPES buffer to between 10^5 to 10^7 particles/μL equivalent to 10^{-13} to 10^{-11} M (approximately 10^8 to 10^{10} haemagglutinin trimers/μL). Aliquots of virus (10 to 25 μL) covering the range of concentrations stated were then injected onto the relevant tracks of the sensor chip fused with either egg yolk lipid or bovine brain lipid. The neomembrane surface was regenerated with 50 mM NaOH. For investigating the effect of temperature (10–35 °C) on binding, the BIAcore instrument was equilibrated for at least 1 h at each temperature before any measurements were made.

4.3. Competitive inhibition experiments

Two concentrations of virus, 3.63×10^{-13} and 1.45×10^{-12} M in buffer containing a range of concentrations of free *N*-acetylneuraminic acid (Sigma) (1.81–22.6 mM) were injected onto the sensor chip. Binding was studied over a range of temperatures from 10 to 30 °C.

4.4. Influence of bacterial neuraminidase (NA) on the binding of virus particles

Solutions of NA (0.02–0.14 mM; Sigma Type V preparation from *Clostridium perfringens*) were passed over the sensor chip at a flow rate of 2 μL/min for 10–25 min both before and after exposure to suspensions of virus. In a second series of experiments the effect of temperature on the action of bacterial NA was studied, again using the enzyme before and after exposure of the sensor chip surface to virus particles.

4.5. Thermodynamic calculations

Influenza virus particles have 1800–3000 potential receptor binding sites (600–1000 HA units) on the surface and it is not possible to determine how many of these bonds are used to form a complex between a virion and a neomembrane surface. Therefore the use of conventional equilibrium thermodynamics to find the equilibrium constant (K_A) is not appropriate since this depends on the number of bonds involved in the formation of a complex. It is possible to investigate the transition state through the rate of association (k_a) and the rate of dissociation (k_d) of a complex through the use of transition state theory (Eyring theory), assuming that we are dealing with a pseudo-first-order reaction.[30] A

recent paper showed that results obtained by application of Eyring theory correlated well with those obtained using conventional equilibrium thermodynamics (van't Hoff theory) for the binding of lysozyme, HIV-1 core protein and a one domain analogue of protein A to immobilised antibodies.[31] Experiments were designed to maintain a low concentration of virus for all kinetic measurements (1.00×10^{-13} M to 6.00×10^{-13}) to limit the concentration of viral NA and therefore side reactions. Moreover, injection times were selected between 150 to 250 seconds to prevent significant accumulation of virus and therefore NA. The selected region of the curve for kinetic analysis was the initial rate between about 50 and 150 s.

Eyring theory says that the rate constant for any reaction is equal to $k_{\aleph}T/h$ multiplied by the equilibrium constant (K^*) for the formation of an activated complex from the reactants.

Thus

$$k = k_{\aleph}T/hK^*$$

where k is the molar rate constant, $k_{\aleph}$ is the Boltzmann constant 1.38×10^{-23} JK^{-1}, T is the absolute temperature and h is the Plank constant 6.63×10^{-34} J s. The term $k_{\aleph}T/h$ is a universal frequency with dimensions of time^{-1} and it is dependent only on temperature and is *independent* of the nature of reactants and the type of reaction.

Thermodynamics say that $\Delta G^* = -RT\ln K^*$ or $K^* = e^{-\Delta G^*/RT}$ where ΔG^* is the free energy of activation. If we rearrange the equation then $k = k_{\aleph}T/he^{-\Delta G^*/RT}$.

Another formulation of the rate equation can be written using

$$\Delta G^* = \Delta H^* - T\Delta S^*$$

ΔH^* is the enthalpy of activation and ΔS^* is the entropy of activation.

The rate equation then becomes $k = (k_{\aleph}T/h)\times e^{-\Delta H^*/RT+\Delta S^*/R}$.

Integrating $\ln(kh/k_{\aleph}T) = -\Delta H^*/RT + \Delta S^*/R$.

A plot of $\ln(kh/k_{\aleph}T)$ versus $1/T$ results in a straight line with a gradient of $-\Delta H^*/R$ and an intercept of $\Delta S^*/R$.

Since $\Delta G_{\text{equilibrium}} = \Delta G^*_{\text{assoc.}} - \Delta G^*_{\text{dissoc.}} = (\Delta H^*_{\text{assoc.}} - \Delta H^*_{\text{dissoc.}}) - T(\Delta S^*_{\text{assoc.}} - \Delta S^*_{\text{dissoc.}})$ it is possible to find a value for the changes in free energy, enthalpy and entropy at equilibrium.

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