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Measurement of kinetic binding constants of viral antibodies using a new biosensor technology

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Association (k_a) and dissociation (k_d) rate constants of three monoclonal antibodies raised against tobacco mosaic virus were determined using a biosensor technique based on surface plasmon resonance (BIAcore, Pharmacia). Dissociation rates were constant over the 4–400 nM antibody concentration range whereas apparent association rates decreased over this range probably due to an increased saturation level of the antigen. Affinity constants K calculated from the ratio of k_a/k_d were in reasonable agreement with values obtained under equilibrium conditions by two standard methods based on enzyme immunoassay.

Key words: Affinity; BIAcore; Biosensor; Kinetics; Monoclonal antibody; Tobacco mosaic virus

Introduction

In order to understand the biological activities of antibody molecules, it is necessary to characterize the strength of their interaction with the antigen in terms of the affinity constant K . In recent studies using different techniques for measuring the antibody affinity of monoclonal antibodies (Mabs) to tobacco mosaic virus (TMV) it was found that operational aspects influenced the experimentally observed affinity values (Azimzadeh et al., 1991, 1992). In some of the techniques the K values were markedly dependent on the antibody concentration used in the assays. When the amount of free antibody present after ultracentrifugation of virus-antibody complexes was measured by enzyme immunoassay, the calculated K value decreased about 25-fold when the

antibody concentration was increased from 30 ng/ml to 35 μ g/ml (Azimzadeh et al., 1992). In the present study, the affinity of several anti-TMV Mabs measured previously by various liquid phase and solid phase methods was determined by a new biosensor technique (BIAcore, Pharmacia, Uppsala) which allows kinetic rate constants of antibodies to be measured. Since the equilibrium affinity constant K is the ratio of the kinetic on and off rates of the reaction, it was of interest to investigate how the kinetic rates were affected by the antibody concentration.

The biosensor instrument (BIAcore, Pharmacia) used in this work is based on surface plasmon resonance, a quantum mechanical phenomenon which detects changes in refractive index in the vicinity of the surface of a thin gold film (Malmquist, 1993). The sensor surface carries a dextran matrix on which one of the immunological reactants is attached. The other is introduced in a flow passing over the surface. Interactions can be followed in real time, so that association

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and dissociation rate constants can be measured and not only the equilibrium constant (Altschuh et al., 1992; Karlsson et al., 1992; Malmberg et al., 1992; Zeder-Lutz et al., 1993).

The study was performed using three monoclonal antibodies (Mabs) specific for the quaternary structure of TMV particles, i.e. three anti-neotope antibodies (Al Moudallal et al., 1985). The results showed that the affinity constants calculated from the ratio of the kinetic rate constants in the BIAcore were in reasonable agreement with the K values determined under equilibrium conditions by two other methods. Dissociation rates were constant over the 4–400 nM antibody concentration range whereas the association rates decreased sharply.

Materials and methods

TMV preparation

Before use, purified virus obtained as described previously was sedimented by ultracentrifugation at $435,000 \times g$ during 5 min (Rotor Beckman TLA 100.2) to remove short particles (Azimzadeh and Van Regenmortel, 1991). The pelleted TMV was resuspended overnight in 10 mM sodium phosphate buffer, 1 mM EDTA pH 7.2. The concentration was adjusted to 1 mg/ml using an extinction coefficient $E_{260} = 3.1 \text{ ml mg}^{-1} \text{ cm}^{-1}$.

Antibody preparation

The three Mabs (79P, 107P, 253P) belong to the subclass IgG1 and are directed against antigenic sites known as neotopes present on the intact quaternary structure of the TMV particle (Al Moudallal et al., 1985; Dore et al., 1988). The antibodies were immunopurified from ascitic fluids as described previously (Hardie and Van Regenmortel, 1977). An extinction coefficient $E_{280} = 1.37 \text{ ml mg}^{-1} \text{ cm}^{-1}$ was found for antibodies 107P and 253P while for antibody 79P it was $E_{280} = 1.39 \text{ ml mg}^{-1} \text{ cm}^{-1}$ (Azimzadeh and Van Regenmortel, 1991).

BIAcore reagents

The instrument (BIAcore) and reagents for interaction analysis were obtained from Biosensor AB, Uppsala, Sweden. The reagents included

the CM5 sensor chips, surfactant P20, *N*-hydroxylsuccinimide (NHS), *N*-ethyl-*N'*-[(3-dimethylamino)propyl] carbodiimide hydrochloride (EDC), ethanolamine-HCl and polyclonal rabbit anti-mouse Fc γ (RAM).

Immobilization on the sensor surface

TMV immobilization. TMV was immobilized on the dextran layer using formic acid buffer at pH 3. Immobilization was achieved by a three step procedure in order to better control the amount of immobilized TMV. About 700–1300 resonance units (RU) of TMV were immobilized by three 10 μl passages at a virus concentration of 20–40 $\mu\text{g/ml}$ using a continuous flow of 5 $\mu\text{l/min}$. A signal of 1000 RU corresponds to a surface protein concentration change of about 1 ng/mm^2 .

Three washes were done after each immobilization to eliminate particles that were not covalently bound. This resulted in a loss of about 100 RU. After each cycle of a run involving antibody binding, the TMV surface was regenerated by a single 25 μl wash of 25 mM HCl. After ten consecutive cycles, a loss of about 20 RU of immobilized TMV was observed.

Mab immobilization. Experiments were done to assess any possible effect on binding kinetics of the direct covalent immobilization of TMV on the matrix. For this purpose, kinetic measurements were made using TMV that was trapped by a Mab bound to a first layer of immobilized rabbit anti-mouse globulin (RAM). The RAM reagent was immobilized according to the manufacturer's instructions. A volume of 35 μl RAM in 10 mM acetate buffer pH 5 at a concentration of 100 $\mu\text{g/ml}$ was injected at a flow rate of 5 $\mu\text{l/min}$, leading to about 13,000 RU of immobilized RAM. Three washes were performed with 25 μl of 100 mM HCl which led to a loss of about 200 RU. A volume of 6 μl of 10 nM 79P was then injected at a constant flow rate of 3 $\mu\text{l/min}$ followed by a saturation step with 3 μl of a control ascitic fluid diluted 1/10. A total of 240 RU of Mab 79P were immobilized. Thereafter 10 μl of TMV (50 $\mu\text{g/ml}$) were injected which led to 1300 RU of bound TMV.

The efficiency of RAM saturation was tested by the same injection sequence except that HBS

(pH 7.4 (10 mM Hepes, 0.15 M NaCl, 3.4 mM EDTA, 0.05% Surfactant P20)) was injected instead of TMV. No significant fixation of antineotope antibody was detected which means that saturation of the RAM was complete.

TMV particles bound in this manner were used to measure the binding kinetics of the three Mabs. After each binding measurement, the surface was regenerated with 100 mM HCl in order to eliminate TMV-antibody complexes from the immobilized RAM. After ten cycles of regeneration on the same sensor chip, a loss of about 90 RU of RAM was observed.

Theory of kinetic measurements

The following symbols are used (Azimzadeh and Van Regenmortel, 1990, Hardie and Van Regenmortel, 1975).

A : total antigen concentration (M^{-1}); B : total antibody concentration (M^{-1}); c : free antigen concentration (M^{-1}); d : free antibody concentration (M^{-1}); y : bound antibody concentration (M^{-1}); n : antibody valence; s : antigen valence; f : ratio of bound antibody to total antigen (y/A); k_a : association rate constant; k_d : dissociation rate constant

The reaction between antigen and antibody can be expressed by $A + B \rightleftharpoons AB$ where the antibody association rate is

$$\frac{d[AB]}{dt} = k_a[A][B] \text{ in } M^{-1}s^{-1} \quad (1)$$

and the antibody dissociation rate is

$$\frac{d[AB]}{dt} = -k_d[AB] \text{ in } s^{-1} \quad (2)$$

Since at equilibrium, $d[AB]/dt = 0$

$$k_a[A][B] = k_d[AB]$$

and $[AB]/[A][B] = k_a/k_d = K$

In our notation, free antigen sites (sc) are expressed as $As - ny$ and free antibody sites are expressed as nd . Thus the reaction rate as a function of free and bound molecules is:

$$\frac{dny}{dt} = k_a^*nd^*(As - ny) - k_d^*ny \quad (3)$$

$$\frac{dny}{dt} = -(k_a^*nd + k_d^*)ny + k_a^*nd^*As \quad (4)$$

In the BIAcore cell, the amount of free antibody is constant since it is continuously being replenished. The resonance units (RU) measured by the instrument correspond to the ny concentration bound to the dextran surface (Fägerstam et al., 1992; Karlsson et al., 1991). For each IgG molecule that becomes bound, two antibody binding sites are immobilized and therefore the valence $n = 2$ should be used in equation 4. Moreover since a low flow rate ($3 \mu\text{l}/\text{min}$) was used, we assumed that both binding sites of each IgG were actually interacting with the antigen (Karlsson et al., 1992; Van Regenmortel and Hardie, 1976). The quantity As was determined from the maximum amount of antibody (Y_{max}) that can be bound to the surface expressed in maximum resonance units (RU_{max}). Equation (4) then becomes

$$\frac{dRU}{dt} = -(k_a^*2d + k_d^*)RU + k_a^*2d^*Y_{\text{max}} \quad (5)$$

When several antibody concentrations are used a linear relationship is obtained between the slope dRU/dt versus RU and the concentration of free antibody sites (nd):

$$\text{slope} = -k_a^*nd - k_d^* \quad (6)$$

From this plot the k_a value is obtained from the slope and the k_d value from the Y intercept.

Measurement of equilibrium K values

In control experiments the affinity constants of each of the three antineotope antibodies were determined using three different techniques: a liquid phase centrifugation method, a solid phase method and the ELISA equilibrium titration method (Azimzadeh et al., 1992). Briefly, in the liquid phase method, a constant amount of antibody ($30 \mu\text{g}/\text{ml}$) was incubated in $200 \mu\text{l}$ centrifuge tubes (Beckman rotor TLA 100) for 1 h at 25°C with variable amounts of TMV ($10\text{--}35 \mu\text{g}/\text{ml}$). Then the virus-antibody complexes were sedimented by ultracentrifugation at $110,000 \times g$ for 10 min and the free antibody concentrations were determined by ELISA.

In the solid phase method, variable amounts of antibody ($1\text{--}50 \text{ ng}/\text{ml}$) were incubated for 2 h in microtiter plates coated with a constant amount

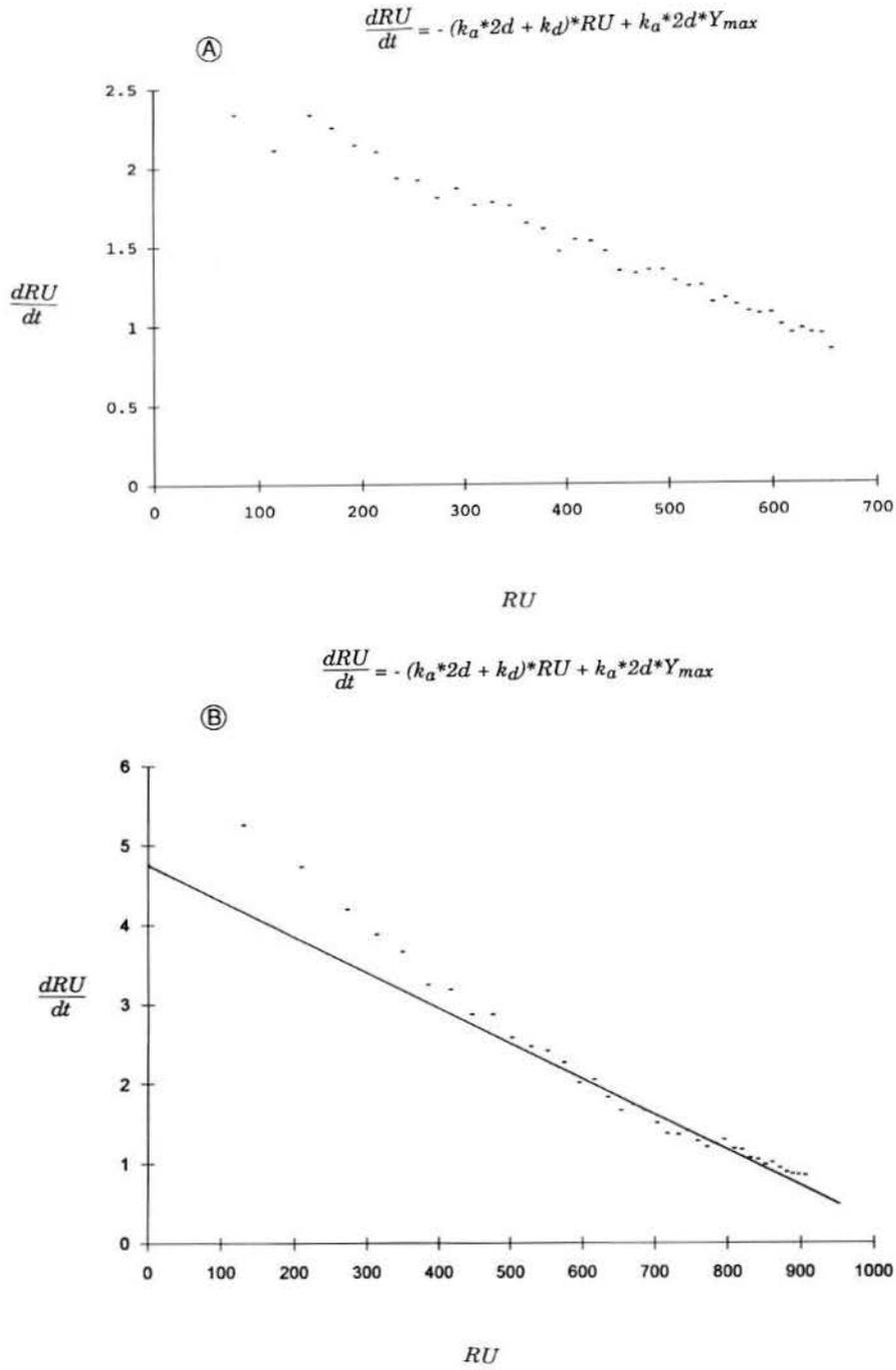


Fig. 1. *A*: plot of dRU/dt vs. RU (equation 5) for antibody 253P (10 nM) binding to immobilized TMV ($R_{max} = 657$). Each mark corresponds to a report point taken every 10 s. The entire range of points was used to calculate k_a . *B*: plot of dRU/dt vs. RU (equation 5) for antibody 253P (8 nM) binding to immobilized TMV ($R_{max} = 1307$). Each mark corresponds to a report point taken every 10 s. Only points below $dRU/dt = 3$ were used to obtain a slope and calculate k_a . *C*: plot of $\ln(R_1/R_n)$ vs. $(T_n - T_1)$ (equation 2) representing the dissociation phase of antibody 253P (10 nM) binding to immobilized TMV. Each mark corresponds to a report point taken every 30 s.

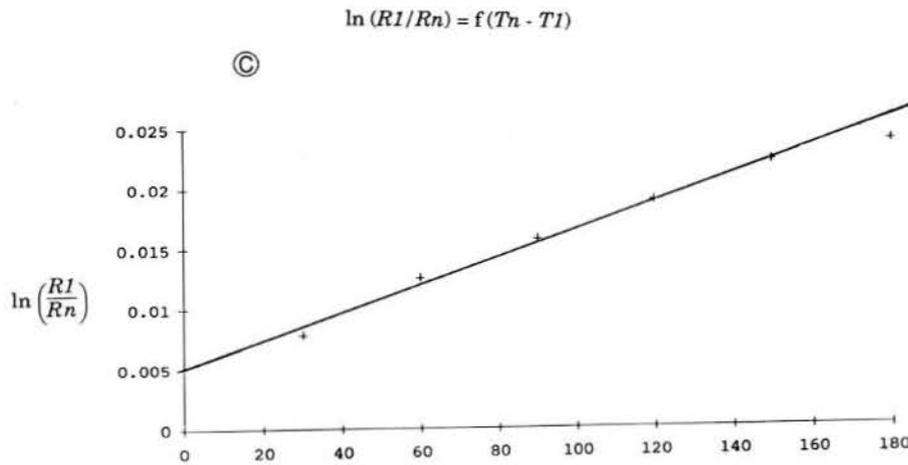


Fig. 1 (continued)

of TMV (0.12 $\mu\text{g/ml}$). Free antibody was removed by simple washing and bound antibody was revealed by means of labelled antiglobulin reagent (Azimzadeh et al., 1992; Frankel and Gerhard, 1979).

In the ELISA equilibrium titration method a constant amount of antibody (50 ng/ml) was mixed with variable amounts of antigen (6–1200 ng/ml). Then the free antibody was measured by ELISA without separating the bound antibody. Data were expressed as $\text{OD}_B - \text{OD}_d / \text{OD}_B$ where OD_B is the OD measured in the absence of antigen and OD_d is the OD measured in the presence of various amounts of TMV (Friguet et al., 1985; Friguet et al., 1989).

Evaluation of kinetic constants

Mabs 79P, 107P and 253P (4–400 nM) in HBS were allowed to interact with immobilized TMV particles. Runs were performed at 25°C, at a flow rate of 3 $\mu\text{l/min}$ taking report points (dRU/dt and RU) every 10 s. The association phase extended over 7 min (21 μl injection of constant antibody concentration); thereafter HBS buffer was continuously injected for the dissociation phase which extended over 3 min. The BIAcore software produces a table containing dRU/dt , RU and t values.

Three different procedures were used for calculating the kinetic constants.

In method 1, only report points of the associa-

tion phase were used; k_a values were obtained as the Y intercept in equation 5 knowing the Y_{max} and d values while the k_d values were calculated from the slope (see Fig. 1A). This method which uses the BIAcore software called BIAevaluation was initially employed (Karlsson et al., 1991, 1992; Altschuh et al., 1992) but is no longer recommended by the manufacturer, mainly because both k_a and k_d values depend on the reliability of the RU_{max} value.

In method 2, kinetic measurements were made at several antibody concentrations. The slopes of each straight line obtained from the plot $dRU/dt = f(RU)$ (equation 5) were used to plot a new curve corresponding to slope = $f(2d)$ where d corresponds to each antibody concentration (Altschuh et al., 1992; Karlsson et al., 1991). In such a plot (equation 6), k_a was obtained from the slope and k_d from the Y intercept.

In method 3, the dissociation constant k_d was obtained from the original result table by plotting $(R_1/R_n) = f(T_n - T_1)$ where R_1 represents the response value in RU at time T_1 and R_n the response values at subsequent times (equation 2). Then, the association report points, corresponding to the corrected response (CorrResp) and the slope (dRU/dt) were plotted using equation 5. Correlation coefficients were used to assess the linearity of each plot. The plots were linear for points below a slope value of 3.0 (Fig. 1A). Points above a slope of 3.0 were not included in the

calculation of k_a (Fig. 1B). Most results described in this study were obtained by this calculation method 3.

Results

Kinetic measurements using immobilized TMV

Kinetic runs were performed on four sensor chips which contained a total of 2155, 3335, 677 and 1307 RU of immobilized TMV. The maximum amount of antibody that could be bound on these four surfaces was 1720, 2944, 600 and 1300 RU respectively. From the ratio of $MW_{\text{antigen}}/MW_{\text{antibody}} = 270$, it follows that a maximum of 270–340 antibody molecules bound to each virus particle. Fig. 2 shows a typical sensorgram illustrating the association and dissociation phases. The marks on the curve correspond to the report points from which the time and slope (dRU/dt) parameters were obtained.

The k_a and k_d values for each antibody were calculated by the three methods described above. The values obtained for antibody 253P by methods 1 and 3 are presented in Table I. The apparent k_a values obtained by method 1 (equation 5) decreased from 2×10^5 to $6 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ when

the antibody concentration increased from 4 to 400 nM.

It appears that the k_a values decrease with increasing degree of saturation of TMV particles, which reflects the portion of the binding curve that yields usable data for the construction of dR/dt vs. R plots. According to equation 5, the initial binding rate k_a obtained from the term $k_a * 2d * Y_{\text{max}}$ should be constant. However, at high antibody concentrations ($f = 250\text{--}270$) the initial binding rate cannot be determined because of sample dispersion and mass transport limitations. The k_d values obtained by method 1 were derived by extrapolation and showed considerable variation. The kinetic values calculated by method 2 were derived from the combination of individual sets of data points measured as in method 1. Data points obtained at antibody concentrations of 2–20 nM were plotted using equation 5. The k_a value was calculated as $4.85 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ and the k_d value as $2.61 \times 10^{-4} \text{ s}^{-1}$ giving $K = 1.85 \times 10^9 \text{ M}^{-1}$. It should be noted that the k_a value obtained by method 2 is higher than the values obtained by method 3 and that the k_d value was also higher than the k_d obtained by method 3. The apparent k_a values obtained by method 3 (Table I) showed the same concentra-

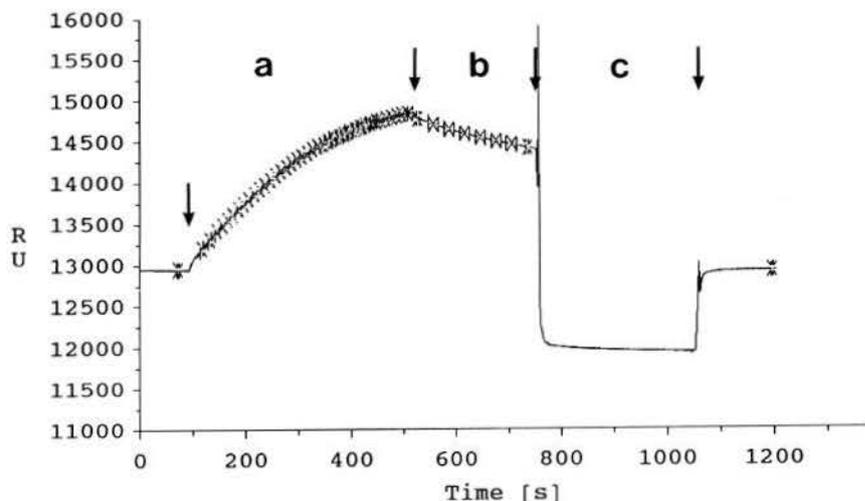


Fig. 2. Sensorgram used for kinetic measurements of Mab 253P when TMV was immobilized. Phase (a) corresponds to the association phase (antibody injection). Phase (b) corresponds to the dissociation phase (buffer injection). Phase (c) corresponds to the regeneration phase with 100 mM HCl ($25 \mu\text{l}$ at $5 \mu\text{l}/\text{min}$). The last report point gives the new baseline for the next measurement.

TABLE I
KINETIC CONSTANTS OF ANTIBODY 253P CALCULATED ACCORDING TO METHODS 1 AND 3^a

Antibody conc. nM	Slope ^b	<i>f</i> ^c	Method 1 ^d			Method 3 ^e		
			<i>k_a</i> 10 ⁵ M ⁻¹ s ⁻¹	<i>k_d</i> 10 ⁻⁴ s ⁻¹	<i>K</i> = <i>k_a</i> / <i>k_d</i> 10 ⁸ M ⁻¹	<i>k_a</i> 10 ⁵ M ⁻¹ s ⁻¹	<i>k_d</i> 10 ⁻⁴ s ⁻¹	<i>K</i> = <i>k_a</i> / <i>k_d</i> 10 ⁸ M ⁻¹
4	-0.00260	130	2.23	8.16	2.73	3.10	1.24	25.0
6	-0.00365	153	2.08	11.5	1.80	2.93	1.34	21.9
8	-0.00464	177	2.11	12.6	1.67	2.81	1.53	18.4
10	-0.00450	179	1.67	11.6	1.44	2.18	1.45	15.0
20	-0.00553	211	1.11	10.9	1.02	1.35	1.52	8.9
30	-0.00496	244	0.72	6.16	1.18	0.80	1.53	5.2
300	-0.00482	270	0.08	- ^f	-	0.08	1.87	0.4
400	-0.00443	268	0.06	- ^f	-	0.05	1.96	0.3

^a Various antibody concentrations were allowed to interact with a sensor chip containing 1307 RU of immobilized TMV.

^b Slope of the straight line obtained according to equation 5.

^c $f = y/A$ where A is the total antigen concentration. f values were determined as the product of (R_{max} at each antibody concentration/RU of immobilized TMV)* (MW of TMV/MW of Ab). A value of $f = 130$ corresponds to $130/270 = 48\%$ saturation level.

^d Method 1 consisted of plotting dRU/dt vs. RU ; the k_a value was obtained from $Y_{intercept}/2*d*Y_{max}$ while the k_d value was obtained by (slope $-Y_{intercept}/Y_{max}$) using equation 5.

^e In method 3 the k_d value was obtained from the dissociation phase of the sensorgram (equation 2) and inserted in equation 5. The k_a value was calculated from the slope of equation 5.

^f k_d values were negative as the slope of equation 5 was lower than the value ($Y_{intercept}/Y_{max}$).

tion dependence as observed in method 1 and are responsible for the observed concentration dependence in the calculated K values. The k_a value corresponding to the average of the last three values obtained at an antibody concentration of 4–8 nM was taken as representative of the kinetic association constant ($k_a = 2.9 \times 10^5$ M⁻¹ s⁻¹). In contrast, the k_d values which in method 3 are obtained from the dissociation phase re-

mained constant over the entire range of antibody concentrations (6–400 nM). The average k_d value was 1.6×10^{-4} s⁻¹ and the calculated K value was 1.8×10^9 M⁻¹.

The kinetic constants of the three antibodies calculated by method 3 as outlined in Table I are presented in Table II. The tenfold higher K value obtained for antibody 253P (at low antibody concentration) compared to the K values of antibod-

TABLE II
KINETIC CONSTANTS OF THREE Mabs MEASURED ACCORDING TO METHOD 3 USING IMMOBILIZED TMV

Antibody	RU of immobilized TMV	Y_{max} ^a RU	f ^b	k_a measurement		k_d measurement		
				Antibody conc.	k_a (10 ⁵ M ⁻¹ s ⁻¹)	Antibody conc.	k_d (10 ⁻³ s ⁻¹)	K ^c (10 ⁸ M ⁻¹)
253P	657	673	276	4–8	4.06 ± 0.62	6–400	0.16 ± 0.03	25.4
253P	1307	1447	299	4–8	2.87 ± 0.20	6–400	0.16 ± 0.02	18
79P	657	575	236	4–10	2.90 ± 0.27	6–400	1.71 ± 0.67	1.7
79P	1307	1250	258	4–10	1.96 ± 0.14	6–400	1.32 ± 0.18	1.5
107P	657	606	249	4–10	3.96 ± 0.47	6–400	1.66 ± 0.05	2.4
107P	1307	1242	257	2–10	2.35 ± 0.18	6–400	1.25 ± 0.20	1.9

^a Y_{max} is the maximum antibody response obtained at a Mab concentration of 400 and 500 nM (60 and 75 μg/ml).

^b $f = y/A$ observed under conditions of antibody saturation (400 nM antibody).

^c Calculated from the ratio k_a/k_d .

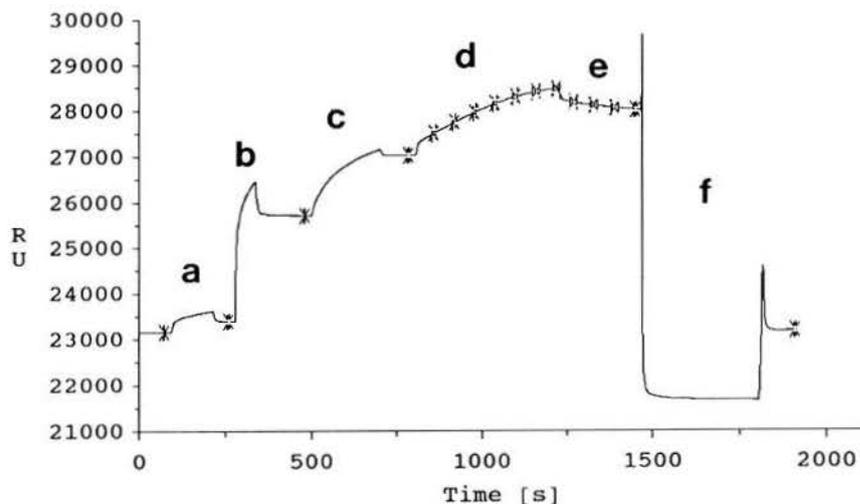


Fig. 3. Sensorgram used for kinetic measurement of Mab 253P with TMV captured by a first antibody. Phase (a) corresponds to the injection of capture Mab 79P (240 RU). Phase (b) corresponds to the saturation of RAM by non specific ascitic fluid (2200 RU). Phase (c) corresponds to TMV injection (1300 RU). Phase (d) corresponds to the association phase (injection of Mab 253P). Phase (e) corresponds to the dissociation phase (buffer injection). Phase (f) corresponds to the regeneration phase with 100 mM HCl (25 μ l at 5 μ l/min).

ies 79P and 107P is shown to be the result of a tenfold lower k_d value (Table II).

Kinetic measurements using TMV captured by a first immobilized antibody

About 1400 RU of TMV were captured by immobilized Mab 79P. When antibodies 79P, 107P and 253P were allowed to bind to the captured TMV, about 2000 RU were bound in all three cases. This corresponds to about 390 antibody molecules bound per virus particle, which is close to the maximum number of antibody molecules that can bind in a bivalent manner in liquid phase

measurements (Azimzadeh and Van Regenmortel, 1991; Van Regenmortel and Hardie, 1976). Fig. 3 shows a typical sensorgram obtained under these conditions. Phase (a) corresponds to the fixation of capture antibody 79P on the RAM while phase (b) represents saturation of RAM with a non-specific Mab. After TMV injection (phase c), the kinetics of association (phase d) and of dissociation of each Mab (phase e) were monitored.

The results obtained with the three antibodies using TMV captured by a first antibody are presented in Table III. For each antibody the k_a

TABLE IV
AFFINITY CONSTANTS OF THREE ANTIBODIES MEASURED BY CLASSICAL AND BIOSENSOR TECHNIQUES^a

Technique	Method	Ab conc. nM	K for 253P 10^8 M^{-1}	K for 79P 10^8 M^{-1}	K for 107P 10^8 M^{-1}
Biosensor	Ratio of k_a/k_d	2–30	20.1 ± 4.5^b	1.5 ± 0.2	2.1 ± 0.3
Enzyme immunoassay	Liquid-phase	200–600	22 ± 3	2.2 ± 1.5	2.5 ± 0.1
	Equilibrium titration	30 or 6	nd ^c	8.8 ± 8	16.1 ± 4.6
	Solid-phase	0.3	30 ± 5^d	7.8 ± 2.7	11.2 ± 5.0
		0.003–0.75	85 ± 30^d	52 ± 18	55.5 ± 20.5

^a All K values correspond to conditions in which bivalent antibody binding predominates.

^b Mean of three values 18.0, 25.4 and 17.0 10^8 listed in Tables II and III.

^c Not determined.

^d From Azimzadeh et al. 1992. All other values in the table were derived from experiments done in the present study.

TABLE III
KINETIC CONSTANTS OF THREE Mabs MEASURED
ACCORDING TO METHOD 3 USING TMV CAPTURED
BY A FIRST ANTIBODY

Antibody	Y_{\max} RU	k_a in $10^5 \text{ M}^{-1} \text{ s}^{-1}$ ^a	k_d in 10^{-3} s^{-1} ^b	K in 10^8 M^{-1}
253	2219	2.90 ± 0.77	0.17 ± 0.27	17
79	1815	1.46 ± 0.24	1.22 ± 0.19	1.2
107	1923	1.76 ± 0.68	0.88 ± 0.22	2

^a Calculated from the k_a values observed at antibody concentration 2–10 nM (0.3–1.5 $\mu\text{g}/\text{ml}$).

^b Calculated from the k_d values observed at antibody concentration 6–400 nM (0.9 \pm 1.5 $\mu\text{g}/\text{ml}$).

value was slightly lower and the k_d value slightly higher than the value obtained with TMV directly immobilized on the matrix.

As found in Table I for antibody 253P, the K values of antibodies 79P and 107P decreased significantly when the antibody concentration increased from 4 to 400 nM (results not shown).

Table IV compares the K values of the three antibodies obtained as k_a/k_d ratios in the BIAcore with the values obtained under equilibrium conditions by three standard techniques. All values pertain to conditions of bivalent antibody binding. The K values obtained, by the liquid-phase and ELISA equilibrium titration methods are in reasonable agreement with the values obtained with the BIAcore. In contrast, K values obtained with the solid-phase method were considerably higher than those obtained by the other methods. Equilibrium K values of Mabs 253P and 107P obtained in this study (Table IV) agree closely with those measured previously by the same techniques (Azimzadeh et al., 1992).

Discussion

Under conditions used for kinetic measurements (3 $\mu\text{l}/\text{min}$ flow rate) a maximum of 270–340 antibody molecules could be bound in a bivalent manner to each TMV particle directly immobilized on the dextran matrix (Table I). In contrast, about 400 antibody molecules could be bound when the virus was trapped by a first antibody. This difference may reflect a better

accessibility of the viral surface when the virus is presented by a first antibody. The lower level of antibody saturation achieved when TMV particles are directly immobilized (Tables I and 2) means that only about 75% of the theoretical number of available antigenic sites per particle ($s = 800$) can be bound by antibodies binding bivalently under these BIAcore conditions. The conclusion that the antibodies were bound in a bivalent manner is corroborated by the finding that the affinity constant K obtained from the ratio k_a/k_d was similar to the values obtained in the case of bivalent binding in liquid phase measurements (Table IV).

A major advantage of the BIAcore technique is that kinetic constants can be obtained without any labelling of the reactants. The k_a values found for the three anti-neotope antibodies were in the range $10^5 \text{ M}^{-1} \text{ s}^{-1}$ while the k_d values varied from 10^{-4} s^{-1} for Mab 253P to 10^{-3} s^{-1} for Mabs 79P and 107P.

The kinetic measurements showed that the association constants should be measured at an antibody concentration above 2 nM since at lower concentration the RU response was small and too variable. At a concentration higher than 30 nM the association phase may be limited by mass transport and biased toward a rapid reassociation and thus lower dissociation facilitated by the high antigenic valence of TMV. The dissociation constant should be obtained from the dissociation phase at an antibody concentration higher than 4 nM since at lower concentrations rapid reassociation also seems to occur. In the concentration range 4–400 nM, k_d values calculated by method 3 were remarkably constant (Table I).

As observed in previous studies of equilibrium K values of antiviral antibodies (Azimzadeh et al., 1991, 1992), we found that the K values calculated from the ratio k_a/k_d were concentration dependent. Table I shows that the apparent association rate constant varied in an inversely proportional manner with the degree of antigen saturation and the concomitant high antibody concentration. Presumably it is the increased saturation level which causes a decrease in association rate and results in the previously observed dependence of K on antibody concentration (Azimzadeh et al., 1991). The decrease in K

value of Mab 253P in the antibody concentration range of 4–400 nM reflects the corresponding decrease in the k_a values since the k_d values remained constant at $1.4 \times 10^{-4} \text{ s}^{-1}$. A similar concentration dependence of K values was observed with Mabs 79P and 107P (results not shown).

For the purpose of comparison, the K values of the three Mabs were again determined in the present study by three classical techniques. As shown in Table IV, the equilibrium K values obtained by the liquid-phase and equilibrium titration methods were in close agreement with the values calculated using the BIAcore. These K values were also similar to those obtained previously by the same techniques (Azimzadeh et al., 1992). It is noteworthy that the solid-phase technique gave K values considerably higher than those obtained by the other methods. This is also in agreement with earlier observations (Azimzadeh et al., 1992) and confirms that the solid-phase technique leads to affinity values that are overestimated 5–10-fold. It has been suggested that the higher K values obtained with the solid-phase technique may be due to the low diffusion rate in microtiter wells leading to a fast antibody reassociation and a decrease in apparent k_d values (Nygren et al., 1987; Sternberg and Nygren, 1988).

Table IV shows that K values derived from kinetic measurements are nearly identical to those obtained in liquid-phase measurements made at high antibody concentration (200–600 nM) but are slightly lower than the values obtained in liquid-phase at low antibody concentration or by the equilibrium titration method. Similar conclusions were reached by Malmborg et al. (1992) who reported that K values obtained with the BIAcore were similar to those found by the equilibrium titration method of Friguet et al. (1985) and were substantially lower than those found by solid-phase methods.

Our results confirm that equilibrium affinity values of anti-viral antibodies calculated from kinetic rates determined with BIAcore are comparable to the values measured under equilibrium conditions by standard procedures. In view of the importance of equilibrium and rate constants for understanding the biological properties of anti-viral antibodies such as their neutralizing

ability, it seems likely that the biosensor technology will find many applications in viral immunology. For instance, it should be of considerable interest to investigate possible correlations between antibody neutralizing ability and the values of the kinetic rate constants. A major advantage of the BIAcore is the relative ease with which kinetic rates can be measured. Although it is to be expected that the values of k_a and k_d measured when one of the reactants is immobilized will differ somewhat from the values measured in liquid phase by, for example, fluorescence measurements (Chaiken et al., 1992), the high reproducibility of measurements made with the BIAcore allows meaningful comparisons to be made between different antibodies. Such comparative measurements should provide useful information for analyzing functional aspects of antibody binding.

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