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Complexes between Monoclonal Antibodies and Receptor Fragments with a Common Cold Virus: Determination of Stoichiometry by Capillary Electrophoresis

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Complex formation between monoclonal antibodies or soluble receptor fragments and a human rhinovirus is quantified by relating the concentration of the antibody or receptor under equilibrium conditions to the initial concentration of the virus. Within a given concentration range of the reactants, the shape of the resulting curve depends only on the value of the dissociation constant of the particular system studied. Using antibodies and receptor fragments, cases for high, low, and intermediate affinity were investigated. For high-affinity systems, the curve approximates a decaying straight line and the binding stoichiometry can be accurately determined from the intercept with the *x*-axis. For the case of intermediate affinity, the curve can be linearized at low virus concentrations with the receptors present in large excess. Extrapolation of this line allows derivation of the binding stoichiometry from the intercept with the *x*-axis, although with less accuracy. For intermediate affinities, an estimate of the dissociation constant can be obtained from fitting the curve to the data points measured. Finally, in the case of low affinity none of the binding parameters can be quantified, although a rough estimate of the lower limit of the dissociation constant is possible. The method was applied for two different monoclonal antibodies, a Fab fragment and a receptor fragment, binding to human rhinovirus serotype 2. Thirty copies of the monoclonal antibody 8F5 were found to bind to the virion, which is in agreement with data from electron cryomicroscopy. The complex between monovalent human very-low-density lipoprotein receptor encompassing repeats 2 and 3 and human rhinovirus serotype 2 showed 60 receptor molecules bound per virion.

Affinity capillary electrophoresis (ACE) has recently emerged as a powerful and effective tool for the determination of parameters underlying various kinds of bioaffinity interactions. It allows direct investigation of the formation of complexes (such as antigen−antibody, enzyme−inhibitor, DNA (RNA)−protein, etc.) by separating them from unbound reactants, setting the stage for the determination of the binding stoichiometry and the thermodynamic equilibrium constants. The underlying theory and its applications have been recently reviewed.[1−5] ACE was used to determine antigen−antibody binding constants in a variety of systems[6,7] and to quantify various compounds by CE-based immunoassays.[8−10] It also permits the measurement of the binding stoichiometry in low- and high-affinity systems by an approach originally proposed by Chu and colleagues.[11] For low-affinity systems, the authors added one of the reactants to the background electrolyte (BGE), thus maintaining equilibrium conditions during the analysis. For high-affinity systems, the reactants were preincubated prior to CE analysis. In these systems, mixtures with fixed concentration of one component (antigen) and increasing concentrations of the other component (antibody) were analyzed and the area of the normalized peak of free antigen was plotted versus the antigen/antibody ratio in the samples. A break in the slope of the plot indicates the stoichiometry of the components. Surprisingly, these are the only published reports on stoichiometry measurements by CE despite a major interest in the ratio of the components in defined complexes, in particular, in systems such as, for example, icosahedral viruses complexed to neutralizing agents. Determination of the stoichiometry allows prediction of the binding topology and might help to localize the binding sites.

Recently, we applied CE for the systematic investigation of macromolecular complexes such as human rhinoviruses (HRVs).[12−17] HRVs, the main causative agents of common cold infections, are icosahedral particles of ∼30 nm in diameter. They

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(1) Rippel, G.; Corstjens, H.; Billiet, H. A. H.; Frank, J. *Electrophoresis* **1997**, *18*, 2175−2183.
(2) Chu, Y. H.; Cheng, C. C. *Cell. Mol. Life Sci.* **1998**, *54*, 663−683.
(3) Kajiwara, H. *Anal. Chim. Acta* **1999**, *383*, 61−66.
(4) Krylov, S. N.; Dovichi, N. J. *Anal. Chem* **2000**, *72*, 111R−128R.
(5) Heegaard, N. H. H.; Nilsson, S.; Guzman, N. A. *J. Chromatogr., B* **1998**, *715*, 29−54.
(6) Heintz, J.; Hernandez, M.; Gomez, F. A. *J. Chromatogr., A* **1999**, *840*, 261−268.
(7) Gomez, F. A.; Avila, L. Z.; Chu, Y.-H.; Whitesides, G. M. *Anal. Chem.* **1994**, *66*, 1785−1791.
(8) German, I.; Buchanan, D. D.; Kennedy, R. T. *Anal. Chem* **1998**, *70*, 4540−4545.
(9) Schmalzing, D.; Nashabeh, W. *Electrophoresis* **1997**, *18*, 2184−2193.
(10) Steinmann, L.; Thormann, W. *Electrophoresis* **1996**, *17*, 1348−1356.
(11) Chu, Y.-H.; Lees, W. J.; Stassinopoulos, A.; Walsch, C. T. *Biochemistry* **1994**, *33*, 10616−10621.

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are assembled from 60 copies each of four different viral proteins (VP1−VP4) and an RNA genome resulting in a molecular mass of $\sim 8 \times 10^6$ Da (for a review, see, for example, ref 18). A very efficient method for the rapid assessment of the purity of rhinoviral preparations was developed which is now routinely used in our laboratory. Further, we applied ACE for the analysis of complex formation between two different virus serotypes, HRV2 and HRV14, and monoclonal antibodies neutralizing the respective serotype. The electrophoretic migration behavior of both serotypes becomes modified upon binding of the cognate monoclonal antibodies; this allowed for identification of affinity complexes and their separation from unbound antibody. These findings set the stage for the determination of binding parameters in virus−antibody and virus−receptor affinity systems.

Using ACE, we here report on binding parameter determination for viruses binding antibodies or soluble receptor fragments. For such systems, the stoichiometry largely exceeds that of molecules analyzed so far with a similar method and can theoretically be within the range of 1:12 and 1:60 (mole of virus/mole of ligand with 12, 24, 30, and 60 symmetry-related equivalent sites present on an icosahedral surface). The topic of the present paper is the determination of the stoichiometry for the reaction of antibodies or receptor fragments with human rhinovirus serotype 2 (HRV2). Binding of these compounds to their recognition sites on the viral surface is assumed to occur independently of the other symmetry-related equivalent sites (noncooperative binding). On the basis of the underlying thermodynamics, the equilibrium concentration of one reactant (antibody or receptor) is determined by the initial concentration of the other reactant (virus) and the thermodynamic equilibrium constant. The magnitude of this constant, together with the initial concentration of antibody or receptor, determines the shape of the curve. Fitting of the thermodynamic function onto the experimental data points allows one to obtain an estimate of the dissociation constant provided that the interaction is stoichiometric and the data points reflect consumption of the monovalent reagent. The possibilities and limitations to derive the binding stoichiometry and the dissociation constant are discussed in detail, and the approach is applied to virus−ligand systems interacting with different affinities.

EXPERIMENTAL SECTION

Apparatus. All experiments were performed with an automated ^{3D}Capillary Electrophoresis System (Hewlett-Packard, Waldbronn, Germany) with a built-in diode array detector. Uncoated fused-silica capillaries, packed in a standard HP cassette and thermostated at 20 °C, were purchased from Composite Metal Service Ltd. Total capillary length was 60.0 cm, effective length 51.5 cm, and i.d. 50 μm. Injection was accomplished by application of 50 mbar pressure to the inlet vial for 9 s. Positive polarity mode with 25 kV was used throughout (negative pole is placed at the capillary outlet). Current generated was typically $\sim$12 μA.

Reagents. HRV2 was produced and purified from infected cell pellets as described previously.[19] Its concentration was determined by CE from the virus peak with corrected virus concentration.[15] Monoclonal antibody (mAb) 8F5, directed against a linear antigenic determinant of VP2, and mAb 3B10, directed against a conformational epitope only present in the context of the viral capsid, were purified from hybridoma tissue culture supernatants, and Fab fragments were prepared by standard procedures.[20] The concentrations of the antibody and the receptor fragment were determined spectrophotometrically by assuming an A_{280} of 13.5 and A_{280} of 14.0 for a 1% solution of the antibody[21] and for the receptor, respectively. The latter value was derived from its amino acid composition.[20,22] All other chemicals were purchased from E. Merck (Darmstadt, Germany) and were used without further purification. Reagent solutions were prepared in deionized water. Buffers were filtered through 0.45-μm cellulose nitrate membranes. All solutions were centrifuged for 2 min in a tabletop centrifuge at 5.000*g* prior to CE analysis.

The BGE was 100 mmol/L boric acid adjusted to pH 8.3 with 1 mol/L NaOH and containing 10 mmol/L sodium dodecyl sulfate (SDS), which is slightly above its critical micelle concentration (8 mmol/L).

Procedures. Between all runs, the capillary was conditioned by flushing with 100 mmol/L NaOH, water, and BGE, for 2 min each, by applying $\sim$950 mbar pressure. All samples were prepared in a buffer, which was 1:2 diluted BGE without SDS added. It contained *o*-phthalic acid (20 μg/mL) as an internal standard (IS).

Determination of Complex Parameters. (1) Monoclonal Antibody 3B10−HRV2. Ten microliters of solution containing mAb 3B10 (2.14 μmol/L) was mixed with equal volumes of HRV2 preparations (from 6.54 nmol/L to 0.105 μmol/L) in microvials, incubated for 1 h at room temperature, and centrifuged at 8000 rpm for 10 min.

(2) Fab Fragments−HRV2. Ten microliters of HRV2 preparation (0.03 or 0.06 μmol/L) was mixed with an equal volume of a solution containing Fab 8F5 (concentration ranging between 0.08 and 5.8 μmol/L) in microvials and incubated at room temperature for 1 h.

(3) Monoclonal Antibody 8F5−HRV2. Ten microliters of solution containing mAb 8F5 (1.2 or 2.4 μmol/L) was mixed with equal volumes of HRV2 preparation (from 8.5 nmol/L to 0.102 μmol/L) in microvials and incubated for 1 h at room temperature.

(4) Receptors−HRV2. Ten microliters of solution containing MBP−VLDLR$_{2-3}$ (3.6 μmol/L) was mixed with an equal volume of HRV2 (concentrations ranging between 11 and 172 nmol/L) in microvials and incubated at room temperature for 1 h. Following the incubations, samples were subjected to CE analysis.

(12) Okun, V. M.; Ronacher, B.; Blaas, D.; Kenndler, E. *Anal. Chem.* **1999**, *71*, 2028−2032.
(13) Okun, V. M.; Blaas, D.; Kenndler, E. *Anal. Chem.* **1999**, *71*, 4480−4485.
(14) Okun, V. M.; Ronacher, B.; Blaas, D.; Kenndler, E. *Anal. Chem.* **2000**, *72*, 2553−2558.
(15) Okun, V. M.; Ronacher, B.; Blaas, D.; Kenndler, E. *Anal. Chem.* **2000**, *72*, 4634−4639.
(16) Okun, V. M.; Moser, R.; Ronacher, B.; Kenndler, E.; Blaas, D. *J. Biol. Chem.* **2001**, *276*, 1057−1062.
(17) Schnabel, U.; Groiss, F.; Blaas, D.; Kenndler, E. *Anal. Chem.* **1996**, *68*, 4300−4303.
(18) Couch, R. B. In *Fields Virology*; Fields, B. N., Knipe, D. M., Howley, P. M., Eds.; Lippincot−Raven Publishers: Philadelphia, 1996; Vol. 1, pp 713−734.
(19) Hewat, E. A.; Neumann, E.; Conway, J. F.; Moser, R.; Ronacher, B.; Marlovits, T. C.; Blaas, D. *Embo J.* **2000**, *19*, 6317−6325.
(20) Tormo, J.; Stadler, E.; Skern, T.; Auer, H.; Kanzler, O.; Betzel, C.; Blaas, D.; Fita, I. *Protein Sci.* **1992**, *1*, 1154−1161.
(21) Kirschenbaum, D. M. *Anal. Biochem* **1973**, *55*, 166−192.
(22) Ronacher, B.; Marlovits, T. C.; Moser, R.; Blaas, D. *Virology* **2000**, *278*, 541−550.

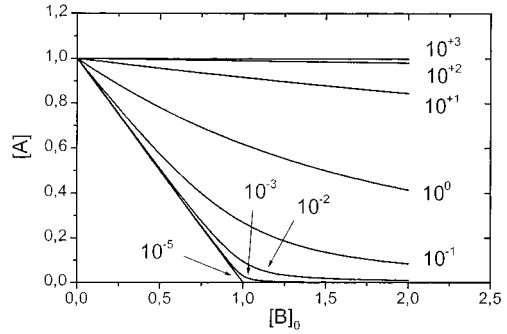

Figure 1. Dependence of the equilibrium concentration of A, [A], on the initial concentration of B, $[B]_0$, for different values of the dissociation constant (between 10^{-5} and 10^3) The function is based on the reaction A + B = AB and is according to eq 4. In the example, the initial concentration, $[A]_0$, is kept constant at a value of 1, and B is added at increasing initial concentrations, $[B]_0$. The concentrations are given in arbitrary units; the dimension of K_d is in the same units.

RESULTS AND DISCUSSION

Basic Thermodynamics of Complex Formation. The equilibrium of formation of the associate AB with 1:1 stoichiometry from the reactants A and B according to

$$A + B = AB \tag{1}$$

is described by the thermodynamic association (or complex) constant, K_a, given as

$$K_a = [AB]/[A][B] \qquad K_d = 1/K_a \tag{2}$$

where the concentrations [A], [B], and [AB] are the equilibrium concentrations of the reactants and the complex, respectively. Note that the dimension of the concentrations and of the dissociation constant, K_d, is mole per liter and that of K_a is liter per mole.

The equilibrium concentrations in the reaction mixture depend on the initial concentrations of A and B, denoted $[A]_0$ and $[B]_0$, and the complex constant. Substituting the relations between the initial and the equilibrium concentrations in eq 1 according to

$$[A] = [A]_0 - [AB]$$
$$[B] = [B]_0 - [AB] \tag{3}$$

gives the equilibrium concentration of A as a function of the initial concentration of the reactants:

$$[A] = [A]_0 - \frac{([A]_0 + [B]_0 + K_d)}{2} \pm \sqrt{\left\{\frac{([A]_0 + [B]_0 + K_d)}{2}\right\}^2 - [A]_0[B]_0} \tag{4}$$

The shape of this function depends on the value of the dissociation constant, K_d related to the concentrations of the reactants.

In Figure 1, the dependence of the equilibrium concentration of A (at an initial concentration $[A]_0$) on the initial concentration of $[B]_0$ (the concentration in the mixture prior to binding to A) is plotted for different values of the dissociation constant according to eq 4. All parameters are given in the same arbitrary units. It can be seen that [A] decreases linearly with increasing concentration of $[B]_0$, when K_d is several orders of magnitude smaller than the analyte concentrations (see, for example, the line for $K_d = 10^{-5}$ arbitrary units). Such a case will be further termed a "high-affinity" system. When the concentration of $[B]_0$ approaches $[A]_0$, both equilibrium concentrations are close to zero with both components having been consumed in the complex. For this high-affinity case, the curve approaches a straight line and the intercept with the *x*-axis gives the stoichiometric concentration of B in the complex. The other extreme case, that of "low affinity", applies for values of K_d of ~2 orders of magnitude larger than the analyte concentrations (see curves with $K_d > 10^2$). Here, the equilibrium concentration of A remains almost unaffected upon addition of B, as reflected by the lines running nearly parallel to the *x*-axis. It must be pointed out again that the ratio between the initial analyte concentration $[B]_0$ and the dissociation constant is decisive for the shape of the curves rather than the value of the equilibrium constant alone.

Between these extreme cases, a range of "intermediate affinity" is found. For example, for K_d values only 1 or 2 orders of magnitude smaller than the initial analyte concentration (10^{-1}–10^{-2} in Figure 1), the curve of $[A]$ vs $[B]_0$ deviates clearly from the straight line. However, even for these cases, the extrapolation of the apparently linear part of the curve at low values of $[B]_0$ allows us to estimate the stoichiometry from the intercept on the *x*-axis. The systematic error of this estimate will increase with an increase of the ratio between dissociation constant and the initial analyte concentration $[B]_0$. Due to the concave shape of the curve, linear extrapolation will result in an underestimate of the binding site number rather than in an overestimate. The error made by extrapolation can be derived from the initial slope of the respective lines at zero $[B]_0$. The bias is 0.2% for a curve drawn for $K_d = 10^{-2}$ (2 orders of magnitude smaller than $[B]_0$) and 10% for a $K_d = 10^{-1}$ (i.e., one-tenth of the analyte concentration). For larger ratios between $[B,]_0$ and K_d, the error increases (it is 53% for $K_d = 5 \times 10^{-1}$), leading finally to unacceptably large deviations. Here, extrapolation of the linear part of the curve to [A] = 0 does not allow for an adequate estimation of the stoichiometry. To circumvent this problem, the concentrations of the components should be increased to the appropriate values. Unfortunately, in many instances, this is not possible due to overloading of the system.

Experimental Determination of the Parameters. As pointed out before, two general ACE approaches based on the kinetics of the interaction studied can be used for binding stoichiometry determination. If the affinity of the interaction is high by the criteria discussed above, the components can be preincubated at various ratios prior to CE analysis. In the case of low affinity, one of the interacting components can be added to the BGE, enabling the maintenance of equilibrium conditions during separation. However, addition of a reactant to the BGE can deteriorate the separation by adsorption to the capillary wall, by decreasing the detection sensitivity due to a high UV absorption, or both. This is especially harmful in the case of proteinaceous compounds. Therefore, precolumn incubation of the reactants is often preferable, provided the complex is stable enough to reach the detector point. If this requirement is fulfilled, the quantification of the

antibody or receptor peak, which decreases upon addition of virus due to complex formation, is taken as the basis for the determination of the stoichiometry. This peak must be well resolved from other peaks. In the case of multivalent systems, such as the icosahedral rhinoviruses, virus and complex peaks can hardly be used for quantification, as complexes with intermediate occupancies cannot be well resolved from each other. However, as reported in our recent papers,[14,15] peaks corresponding to antibodies or recombinant receptor fragments can be satisfactorily separated from the other components (free virus and complexes) permitting their quantification. Since significant dissociation would deteriorate correct estimation of stoichiometry, the stability of the complexes was studied separately. We demonstrated that equilibrium conditions were attained at 60 min of incubation with no appreciable dissociation of the complexes within the time of analysis;[14] the latter was obvious from the peak symmetry of the free reagent. On the basis of these findings, antibodies or receptor fragments were reacted with HRV2 by precolumn incubation and the equilibrium concentrations of the analytes in the reaction mixture were subsequently determined by CE under nonequilibrium conditions.

In the following, we assume that all sites on the virus surface are equivalent with respect to their interaction with the ligands; i.e., ligands attach to n equivalent epitopes with equal affinity (noncooperative binding). Under this assumption, the reaction between one antibody molecule (with its two Fv arms) and two of the symmetry-related equivalent sites (as seen from the complex between monoclonal antibody 8F5 and HRV2[23]) is considered a 1:1 reaction (although an antibody reaction is not necessarily bidentate; see, for example, ref 24). For a critical discussion of the concept, see, for example, ref 25. The interaction is described by eq 1, where A stands for antibody or receptor and B for the binding sites on the viral surface reacting with one single antibody or receptor (in most cases, this will be one or two sites). On each virion, n such sites are available for ligand attachment. From this it follows that B_0 mol of virus are counted as nB_0 mol of binding sites.

Binding Curves. Two different virus neutralizing monoclonal antibodies (8F5,[26] 3B10 [24]), a Fab fragment of 8F5, and a recombinant soluble receptor fragment derived from the very-low-density lipoprotein receptor (VLDLR)[22] were used as reactants for complex formation with HRV2. Whereas 8F5 binds bivalently over the 2-fold axes of icosahedral symmetry, the geometry of the binding sites recognized by 3B10 does not allow bidentate binding and this antibody neutralizes by aggregation of several virions.[24] Its neutralization efficiency compares well to that of other aggregating monoclonal antibodies directed against picornaviruses (see, for example, ref 27). The monovalent Fab fragments, however, bind to the 60 equivalent sites present on one virion.[24] Finally, on the basis of earlier findings with CE analysis,[16] it was assumed that 60 VLDLR fragments would also bind to a single virion. Binding curves were established in order to first examine whether their shape would allow for the determination of the stoichiometry, and in cases of intermediate affinity, an attempt was made to derive the dissociation constant for the virus−receptor complex.

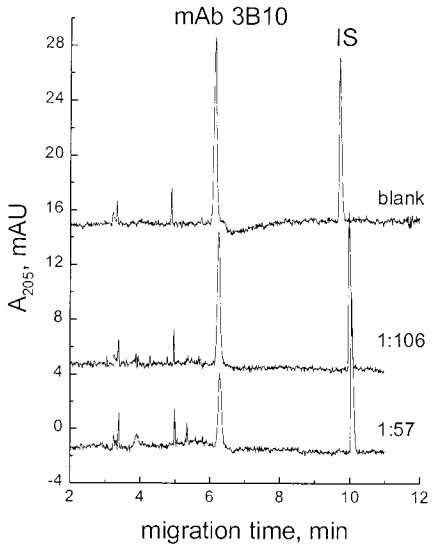

Figure 2. Electropherograms from mixtures of mAb 3B10 with HRV2. Upper trace: mAb 3B10 at 1.07 μmol/L. Middle and lower traces: mixtures of antibody with HRV2 at 106 and 57-fold molar excess of mAb over HRV2. Conditions: capillary 60.0 (51.5) cm length, 50 μm i.d.; BGE 100 mmol/L borate/boric acid buffer, 10 mmol/L SDS, pH 8.3; voltage +25 kV; IS, internal standard, *o*-phthalic acid.

High-Affinity and Low-Affinity Systems. *Monoclonal Antibody 3B10.* For generation of the binding curve, a constant amount of 3B10 was incubated with increasing amounts of HRV2 for 1 h at room temperature and aggregates were removed by centrifugation at 8 krpm for 20 min. Under these conditions, single virions are not pelleted and remain in the supernatant.[12,13] The decrease of the antibody peak with increasing virus concentration is clearly seen in Figure 2. No peak corresponding to free virus or to antibody−virus complexes is apparent, indicating that all of the virus was aggregated and removed by centrifugation. The corresponding antibody peak areas, normalized to the internal standard, plotted versus the initial HRV2 concentration are given in Figure 3 (main plot). This correlation is linear ($r = 0.999$) up to the very last point determined, indicating a high-affinity interaction (compare to Figure 1). As long as antibody-binding sites are available on the viral surface, the antibody will be removed from the mixture. Indeed, although this system is heterogeneous, the virus is precipitated by antibodies with a stoichiometry of the components of 1:18. This value was highly reproducible in several experiments, and the ratio most probably reflects the accessibility of the antibody binding sites as dictated by steric hindrance. From the linear appearance of the curve it can be deduced that we are dealing with a high-affinity interaction (see Figure 1).

At the intercept of the curve with the x-axis, the condition is just met where all antibody has been consumed by the virus; i.e., virus and antibody are present in a stoichiometric complex. As the x-axis is scaled in units representing the number of virus particles initially present in the mixture per antibody molecule,

(23) Hewat, E. A.; Blaas, D. *Embo J.* **1996**, *15*, 1515−1523.
(24) Hewat, E. A.; Marlovits, T. C.; Blaas, D. *J. Virol.* **1998**, *72*, 4396−4402.
(25) Junghans, R. P. *Immunol. Today* **1999**, *20*, 401−406.
(26) Skern, T.; Neubauer, C.; Frasel, L.; Gruendler, P.; Sommergruber, W.; Zorn, W.; Kuechler, E.; Blaas, D. *J. Gen. Virol.* **1987**, *68*, 315−323.
(27) Mosser, A. G.; Leippe, D. M.; Rueckert, R. R. In *Molecular Aspects of Picornavirus Infection and Detection*; Semler, B., Ehrenfeld, E., Eds.; American Society for Microbiology: Washington, DC6, 1989; Vol. 1, pp 155−167.

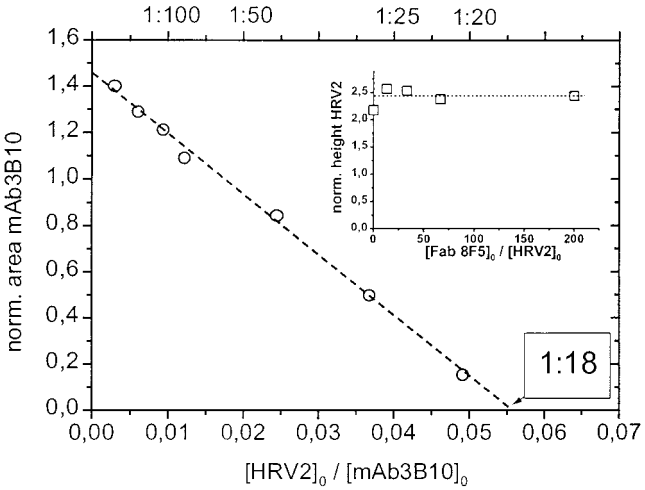

Figure 3. Examples of high- and low-affinity interactions. The peak areas of the free antibodies mAb 3B10 in the reaction mixture after incubation with HRV2 at different initial concentrations are plotted vs the initial concentration of the antibodies (see Figure 2 for three points out of the six used here). On the upper axis, the molar ratios of virus/antibody are indicated. All peak areas were normalized to the internal standard. The linear correlation of the data points is 0.999 for both cases. Inset: HRV2 was incubated at a constant amount with 8F5 Fab at different initial concentrations, and the peak height of HRV2 after incubation was determined by CE. For details, see text.

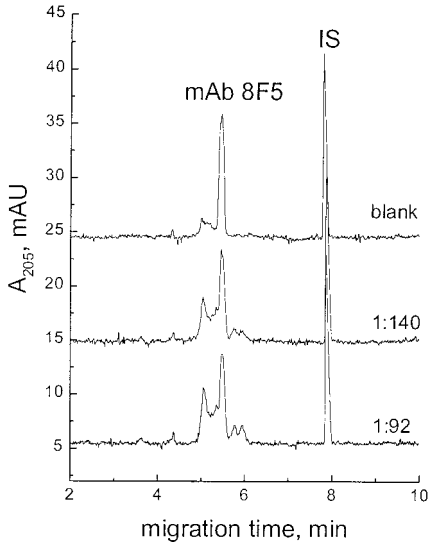

Figure 4. Electropherograms obtained after establishment of the equilibrium between mAb 8F5, HRV2, and their complex. Upper trace, mAb 8F5 at 1.2 μmol/L; middle and lower traces, mixtures with a 140- and a 92-fold molar excess of mAb 8F5 over HRV2. Conditions as in Figure 2. IS, internal standard.

the value of x at $y = 0$ gives the number of antibody molecules bound per single virion.

8F5 Fab Fragments. Cryoelectron microscopy revealed that the occupancy, i.e., the number of epitopes bound by 8F5 IgG, was high whereas Fab fragments of the same antibody only bound occasionally.[23] Apparently, under equilibrium conditions, one Fv arm of the antibody might dissociate from the viral surface with the other still remaining bound; thereby, reassociation is facilitated. The affinity of the 8F5 Fab fragment for its cognate epitope is thus clearly less than the avidity (see above) of the bivalent antibody which attaches to two symmetry-related epitopes more or less simultaneously (for discussion of the interaction of this antibody with HRV2, see ref 23).

Fab fragments of 8F5 were incubated with two initial concentrations of HRV2 in two independent experiments, and the equilibrium concentration of HRV2 was measured by CE. As seen in Figure 3, the normalized peak height (the ratio between the peak height of the analyte and that of the internal standard) of HRV2 in the incubation mixture remained constant up to a molar excess of the Fab fragments of 200 over HRV2 (Figure 3, inset). Clearly, complexes did not form under these conditions, indicating too low affinity. For obvious reasons, no value for the stoichiometry can be derived although, at very high Fab concentrations, 60 fragments are expected to be bound per virion. A value for the dissociation constant cannot be deduced under these conditions. Based on analyte concentrations of the order of 10^{-6} mol/L, the dissociation constant must be higher than 10^{-4} mol/L as beyond this value the curve should substantially deviate from the horizontal line given that the concentrations of the analytes are 100 times less (see Figure 1).

Intermediate-Affinity Systems. Systems with dissociation constants within the range of the analyte concentrations as well as 1 or 2 orders of magnitude smaller result in typical concave [A] vs $[B]_0$ curves as shown in Figure 1. In these particular cases, the stoichiometry of the components in the complex can be determined with reasonable accuracy. Moreover, an estimation of the extent of deviation from the true value can be made. This is of major importance since the error is biased toward lower numbers of antibody bound, and in most cases, it is necessary only to decide between some clearly defined values dictated by the viral symmetry. In the easiest case, distinction between bivalent and monovalent attachment comes down to a decision between a stoichiometry of 1:30 or 1:60.

Monoclonal Antibody 8F5. mAb 8F5 was incubated with increasing concentrations of virus, and the samples were analyzed by CE. In total, electropherograms from seven different analyte concentrations were obtained and three examples from this set are shown in Figure 4. A decrease of the antibody peak with a concomitant appearance of the complex peak (migration time 5.1 min) can be clearly seen upon HRV2 addition. Recently we observed[15] that IgG−HRV2 complexes once formed are stable enough to see no dissociation for at least up to 1 h of incubation under the conditions used.

The normalized peak heights of IgG 8F5, as derived from CE measurements, were plotted versus the initial virus concentration in the mixtures. The resulting curve (Figure 5) exhibits a shape similar to those derived for K_ds in the range of 10^0−10^{-2} arbitrary units (see Figure 1). It is divided into a concave and a linear part (the latter at low virus concentrations), which allows extrapolation to zero concentration of free antibody (intercept with x-axis; see dots). The stoichiometry derived from these data for the complex IgG 8F5−HRV2 was 1:28, which is in good agreement with the topology of attachment of this antibody as seen from the reconstruction of the cryoelectron microscopy images obtained from the complexes.[23] An almost identical result was obtained when the initial concentration of IgG was doubled (Figure 5, lower curve). Both results are clearly compatible with an expected stoichiometry of 1:30.

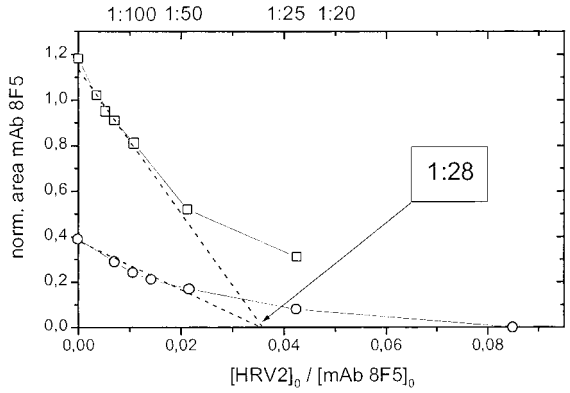

Figure 5. Estimation of the stoichiometry for intermediate-binding affinity: HRV2/mAb 8F5. The concentration of mAb 8F5 in the reaction mixture (initial mAb 8F5 concentrations of 1.2 (□) and 0.6 μmol/L (○)) as a function of the initial concentration of HRV2 (given as manifolds of the initial antibody concentration). Peak areas corresponding to mAb 8F5 (see Figure 4) were normalized to the peak areas of the IS. The molar ratios of virus/antibody are indicated on the upper axis. The dotted lines are the extrapolation of the linear part of the curves to zero antibody concentration indicating 1:28 complex stoichiometry from the intercept of the *x*-axis.

MBP−VLDLR$_{2-3}$ Receptor Fragments. Recombinant soluble fragments of the low-density lipoprotein receptor[28,29] and the VLDLR[22] have been evaluated as competitive inhibitors of viral infection. In fact, the absence of steric hindrance allows one VLDLR$_{1-3}$ fragment to bind to one single viral epitope;[19] the system thus obeys the conditions under which eq 1 is valid. One of the smallest VLDLR fragments active in virus binding encompasses the second and third ligand binding repeats fused to the C-terminus of the maltose binding protein to aid bacterial expression.[22] A stoichiometry of 1:60 for a larger fragment with repeat 1−3 has been recently determined by CE[16] and confirmed by electron cryomicroscopy image reconstruction techniques.[19] Stoichiometry determination was attempted for the smaller fragment, termed MBP−VLDLR$_{2-3}$, in the present work. Three electropherograms from a set of seven obtained after incubation of MBP−VLDLR$_{2-3}$ with increasing concentration of HRV2 are shown in Figure 6. It can be seen that the fragment peak decreases upon addition of HRV2, and a well-separated and broad peak appears at a migration time of ∼5 min; this seemingly corresponds to the heterogeneous complex. No dissociation is seen, at least within the time scale of analysis, which is obvious from the symmetry of the receptor peak. The stability of complexes between HRV2 and different kinds of receptors was studied recently.[16]

The dependence of the equilibrium concentration of MBP−VLDLR$_{2-3}$ on the initial HRV2 concentration is depicted in Figure 7. The intercept with the *x*-axis from the linear extrapolation of the curve at low virus concentrations indicates a stoichiometry of 1:43. However, from the shape of the binding curve, it can be expected that this extrapolation leads to an underestimation (see above). On the basis of the topology of an icosahedron, only distinct combinations of virus and receptor fragment are plausible.

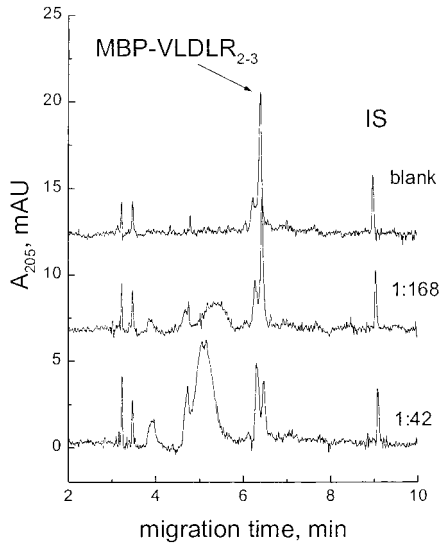

Figure 6. Electropherograms from mixtures of MBP−VLDLR$_{2-3}$ with HRV2. Upper trace, MBP−VLDLR$_{2-3}$ at 1.8 μmol/L; middle and lower traces, mixtures with a 140- and a 92-fold molar excess of mAb 8F5 over HRV2. Conditions as in Figure 2. IS, internal standard.

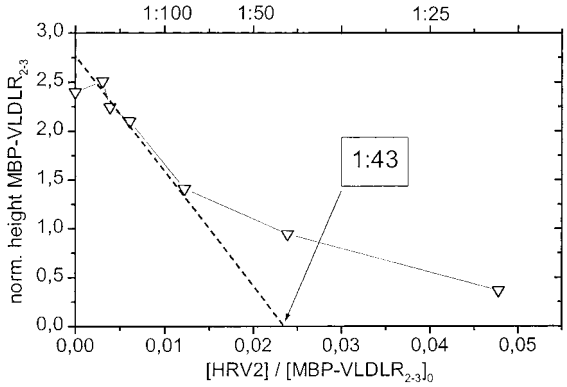

Figure 7. Estimation of the stoichiometry for intermediate binding affinity: HRV2/MBP−VLDLR$_{2-3}$: the concentration of the soluble receptor fragment MBP−VLDLR$_{2-3}$ in the reaction mixture as a function of the initial concentration of HRV2 (given as manifolds of the initial antibody concentration). Peak areas corresponding to MBP−VLDLR$_{2-3}$ were normalized to the peak areas of the IS.

Assuming that 43 is an underestimate and the next larger value is 60, the most likely stoichiometric composition of the complex is 1:60.

In previous publications, a similar approach was applied for stoichiometry determination in high- and intermediate-affinity systems, namely, for biotin and biotin-binding protein (high affinity[30]) and for VLDLR$_{1-3}$ or VLDLR$_{1-8}$ and HRV2 (intermediate affinity[16]). Curves generated identically as in the present paper fit the theoretical model presented here (Figure 1) and the derived stoichiometry correlated with theoretical expectations.

Dissociation Constants of Virus−Receptor Complex. It was pointed out above that intermediate-affinity cases allow one to derive the stoichiometric composition with less accuracy than high-affinity systems. On the other hand, provided that the reagent under study binds monovalently, the dissociation constant can be determined from fitting the function to the measured concentration values. Thus, in an attempt to determine the dissociation constant

(28) Marlovits, T. C.; Abrahamsberg, C.; Blaas, D. *J. Virol.* **1998**, *72*, 10246−10250.

(29) Marlovits, T. C.; Zechmeister, T.; Gruenberger, M.; Ronacher, B.; Schwihla, H.; Blaas, D. *FASEB J.* **1998**, *12*, 695−703.

(30) Okun, V. M.; Bilitewski, U. *Electrophoresis* **1996**, *17*, 1627−1632.

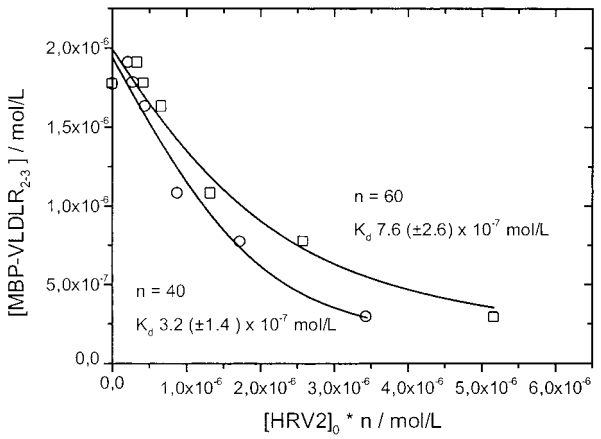

Figure 8. Determination of the affinity constant for the interaction between receptor fragment MBP−VLDLR$_{2-3}$ and HRV2 by curve fitting according to eq 4. The equilibrium concentrations of the receptor fragments (as derived from the electrophoretic peak areas) were plotted vs the initial concentration of the binding sites on HRV2 based on a number, n, of 60 and 40, respectively, available binding sites per virus. The estimate of the standard error, σ_i, is calculated from χ^2 and from the diagonal element, C_{ii}, of the variance−covariance matrix, according to $\sigma_i = \sqrt{(C_{ii}\chi^2)}$. The dotted lines indicate the 95% confidence interval of the fitted curve.

of the complex between MBP−VLDLR$_{2-3}$ and HRV2, the data points (Figure 5) were fitted to eq 4 using the concentration of the binding sites ($n[B]_0$) rather than the concentration of the virus ($[B]_0$, see above). Using a stoichiometry of 1:60 (the most plausible value following the underestimated value of $n = 43$ obtained from the experiment), the complex constant was derived from curve fit to the data points (Figure 8). On the basis of eq 4, a value of 8×10^{-7} mol/L for the dissociation constant was obtained. For sake of comparison, the curve was also calculated by assuming the less plausible number of 40, also shown in Figure 8. From this results a K_d of 3×10^{-7}. This shows that the value of the dissociation constant is not very much influenced by the above assumptions and demonstrates that the method allows for an estimate of its value.

The dissociation constant derived for the receptor is ~1 order of magnitude lower than the values obtained for similar systems such as major group rhinoviruses and their receptor, intercellular adhesion molecule-1 (ICAM-1)[31] and echovirus 11, and its cellular receptor, CD55[32] as determined by surface plasmon resonance (SPR) technology. Work currently in progress will show whether our results correlate with SPR data for minor group rhinovirus and VLDL receptor fragments.

CONCLUSIONS

On the basis of the thermodynamics of the formation of complexes between a human rhinovirus and neutralizing ligands, the extent of the affinity reaction was quantified by relating the equilibrium concentration of one reactant to the initial concentration of the other reactant added to the incubation mixture. Taking into account the presence of equivalent sites related by the icosahedral symmetry of the virion, this equation describes the reaction between one molecule of the receptor and the equivalent binding sites, assuming that the interaction is independent of the presence of the other sites. Plots based on different numerical values for the dissociation constant and the initial concentrations of the reactants resulted in curves of characteristic shapes, which depend on the relation between the dissociation constant and the analyte concentration. From this, three types were distinguished representing high, intermediate and low affinity.

Several systems were explored experimentally by the determination of the equilibrium concentration of antibody or receptor by CZE from the peak area. For the high-affinity case, the stoichiometric complex composition is obtained from plots of the data points versus the initial virus concentration at the intercept of the decaying straight line with the x-axis. For intermediate-affinity systems, the binding stoichiometry can also be derived, however, with less accuracy dictated by the affinity and the initial concentrations of the analytes. For the low-affinity case, obviously no stoichiometry can be derived.

The stoichiometric composition was determined for the complex of a nonaggregating antibody (mAb 8F5) and of the soluble receptor fragment of MBP−VLDLR$_{2-3}$ with HRV2. For the latter intermediate-affinity system, the dissociation constant was determined to be within the range of several hundred nanomoles per liter.

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(31) Casasnovas, J. M.; Springer, T. A. *J. Biol. Chem.* **1995**, *270*, 13216−13224.
(32) Lea, S. M.; Powell, R. M.; McKee, T.; Evans, D. J.; Brown, D.; Stuart, D. I.; vanderMerwe, P. A. *J. Biol. Chem.* **1998**, *273*, 30443−30447.