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Competition BIAcore for Measuring True Affinities: Large Differences from Values Determined from Binding Kinetics

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In attempting to use the BIAcore instrument for the determination of binding constants of several haptens or peptides to different antibodies by measuring on- and off-rates, we found that neither the absolute nor the relative values of the binding constants corresponded to the measurements in solution. Even at the lowest coupling densities useful for measurements, rebinding and bivalency effects offset the measurements by a factor of up to 500. We caution therefore about using on- and off-rates for the determination of absolute or even relative binding constants without controlling for rebinding and avidity effects. Instead, we show that binding constants in solution can be reproduced well by using on-rate determinations of antibody preincubated with antigen, and we derive the conditions under which such an approach is valid.
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Optical biosensors, such as the BIAcore instrument, are powerful tools for the analysis of biomolecular interactions and their applications are expanding rapidly (1–4). The main advantage is that no particular molecular property (such as fluorescence or radioactive label) in either of the interacting molecules is required. In principle, optical biosensors monitor the binding of macromolecules in real time and therefore give more information than just the binding constant. Nevertheless, a number of assumptions are inherent to the method, which when not fulfilled skew the data and limit its utility when used in the standard way.

In the BIAcore instrument, changes in the refractive index, close to the sensor surface, are monitored using surface plasmon resonance detection (5). Stenberg and co-workers (6) showed that for proteins changes in the refractive index are proportional to the accumulation of mass. In the particular setup of the BIAcore instrument, the binding events occur in a three-dimensional carboxymethylated dextran matrix, to which the ligand is covalently attached. In a typical experiment, the analyte is injected into a flow cell over the surface and the change in refractive index, which indicates binding, is recorded versus time to record the association phase. In the dissociation phase the analyte is washed out by buffer and the loss of bound analyte from the sensor is monitored as a function of time. These data are used to calculate kinetic constants and thus equilibrium constants for the interaction between the analyte and the ligand (7, 8). Inherent in the method is the assumption that these are true association and dissociation rates for a one-to-one interaction model.

In attempting to analyze the binding properties of several haptens to antibodies from determining on- and off-rates as described above, we observed great discrepancies with values obtained in solution. We present evidence that the off-rate especially does not reflect the microscopic dissociation rate. We therefore tested and refined an approach recently described by Karlsson (9), which essentially measures equilibrium binding in solution, yet uses the kinetic data from the BIAcore instrument to monitor binding. With this approach one can now determine monomeric binding constants (intrinsic thermodynamic affinities) and multivalent surface binding (functional affinities) in the same experiment, and this can be combined with an epitope mapping study in solution.

The current methods of epitope mapping for haptens using biosensors are unsatisfactory. Similarly, the binding of small molecules to receptors, a process of immense interest to pharmaceutical research, suffers from the same limitations. If the antibody is coupled, the mass of the hapten is too small for detection. If the haptens are coupled, a new chip has to be used for each experiment, and a very well controlled coupling density has to be achieved, because of the rebinding phenomena during the dissociation phase, which occur both

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for monovalent and for bivalent molecules (see below). Furthermore, avidity effects from bivalent binding may obscure the intrinsic thermodynamic affinities of the single binding site when using bivalent antibodies. Since it is not possible to have precisely identical coupling densities for different haptens, direct comparisons of affinities are essentially impossible. Furthermore, we show here that even *relative* on/off-rate ratios, because of the extraordinarily complex nature of rebinding and multivalency effects, do not directly reflect relative thermodynamic affinities.

MATERIALS AND METHODS

Instrumentation and reagents. The BIAcore system and reagents including sensor chips CM5, surfactant P20, 2-(2-pyridinyldithio)ethaneamine hydrochloride (PDEA),[2] and the amine coupling kit containing *N*-hydroxysuccinimide (NHS), *N*-ethyl-*N'*-(3-diethylaminopropyl)carbodiimide (EDC), and ethanolamine hydrochloride were obtained from Pharmacia Biosensor AB (Uppsala, Sweden). MBS (3-maleimidobenzoic acid *N*-hydroxysuccinimide ester) was obtained from Fluka. Ampicillin was obtained from Sigma, D-phenylglycine methylester was obtained from Bachem. Benzoylampicillin was synthesized from NHS-activated benzoic acid and ampicillin, purified by HPLC and analyzed by ^{1}H-NMR and mass spectroscopy. The peptide Lys-Gly-Gly-His-His-His-His-His, used for the BIAcore experiments, was synthesized using standard solid-phase methods and analyzed by mass spectroscopy and amino acid analysis. The buffer used for all experiments was HBS (10 mM Hepes, 3.4 mM EDTA, 150 mM NaCl, 0.05% surfactant P20, pH 7.4).

Antibodies. The hybridoma cell line producing the antibody 2H10 (10) was a kind gift from Dr. W. Stimson (Strathclyde, UK) and the hybridoma cell line producing the anti-His-tag antibody (K. Bauer, E. Kremmer, and R. Mocikat, unpublished) was a kind gift from Dr. Mocikat (GSF, München, Germany). The antibodies were purified from hybridoma culture with a protein G High Trap column (1 ml) (Pharmacia). The concentration of the purified 2H10 antibody was then determined from the absorbance at 280 nm ($\epsilon_{1\ mg/ml} = 1.574$; MW 144,500 Da) (11). The concentration of the anti-His-tag antibody was determined using the bicinchoninic acid assay (Pierce).

The scFv fragment of the anti-His-tag antibody was cloned, expressed and purified from *Escherichia coli* raw extract (12) using a one-step affinity chromatography on immobilized oligo(His) peptide. The concentration was determined by absorbance at 280 nm (11). A different scFv fragment, specific for ampicillin and carrying a C-terminal His-tag, was used as antigen for the anti-His-tag antibody and coupled to the sensor chip for determination of the K_D of the anti-His-tag antibody. It was purified by affinity chromatography on immobilized ampicillin.

ELISA and competition ELISA. The different penicillin antigens were coupled to the MBS linker exactly as described for BIAcore (see *Immobilization of ligands onto the sensor surfaces*) and then covalently attached to free SH groups in reduced and denatured transferrin (0.2 μmol human transferrin, obtained from Sigma, in 1 ml 0.05 M sodium phosphate, pH 7.0, 8 M urea). One hundred microliters per well of a 1:100 dilution of the transferrin-coupled antigen solution [diluted in PBS (phosphate-buffered saline)] was incubated overnight at 4°C on a microtiter plate. Blocking was achieved with 5% milk in PBS for 2 h at room temperature. The ELISA was carried out both with hybridoma supernatant and with purified antibody (10 and 80 nM). Detection was achieved with an anti-mouse-Fab antibody coupled to horseradish peroxidase. Both the first and the detection antibody were incubated on the ELISA plate for 1 h. The colorimetric reaction (BM blue substrate, soluble; Boehringer Mannheim) was then measured in an ELISA reader (Dynatec) at 405 nm. Inhibition ELISAs were carried out by preincubation of the antibody with the antigens at different concentrations for 10 min at room temperature.

Immobilization of the ligands onto the sensor surfaces. Immobilization of haptens for the anti-β-lactam antibody 2H10 was performed as follows: (a) Equal volumes of NHS (55 μl, 0.06 M in water) and EDC (55 μl, 0.2 M in water) were first mixed and then 25 μl of this solution was pumped across the chip to activate the carboxymethylated dextran surface. (b) Twenty microliters of cystamine hydrochloride (40 mM) were pumped across the activated surface. (c) Residual NHS esters were inactivated with ethanolamine (20 μl, 1 M, pH 8.5). (d) To obtain free SH groups on the surface, 20 μl DTT (0.1 M) was injected. (e) The flow was reduced from 5 to 2 μl/min. (f) MBS–ampicillin, MBS-hydrolyzed ampicillin, or MBS-D-phenylglycine methylester were prepared beforehand from MBS (10 mmol dissolved in 0.25 ml THF) and the corresponding ampicillin derivative (10 mmol dissolved in 0.75 ml sodium phosphate buffer, 50 mM, pH 7.0). This solution [10^{-2} M MBS–ampicillin derivative in sodium phosphate buffer (37.5 mM, pH 7.0) and 25% THF] was incubated for 30 min at 30°C and finally diluted with 50 volumes HBS buffer to reduce the amount of THF to under 1%, and then 20 μl of this solution was in-

[2] Abbreviations used: NHS, *N*-hydroxysuccinimide; MBS, 3-maleimidobenzoic acid *N*-hydroxysuccinimide ester; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; EDC, *N*-ethyl-*N'*-(3-diethylaminopropyl)carbodiimide; DTT, dithiothreitol; PDEA, 2-(2-pyridinyldithio)ethaneamine hydrochloride; GdnCl, guanidinium hydrochloride; DPG-OMe, D-phenylglycine methylester; THF, tetrahydrofuran.

jected. (g) The flow was increased to 5 μl/min. (h) Residual SH groups were blocked with 10 μl PDEA solution (20 mM PDEA, 1 M NaCl, pH 4.3).

Two surfaces were used for determining the K_D of the anti-His-tag antibody. For the first, the peptide Lys-Gly-Gly-His-His-His-His-His was coupled to the dextran matrix via standard amine coupling chemistry using the amine coupling kit. For the second surface, a protein carrying a C-terminal His-tag (scFv fragment specific for ampicillin) was coupled to the dextran matrix in a random orientation by using standard amine-coupling chemistry.

Binding assays and data analysis. Each binding cycle was performed with a constant flow of HBS of 5 or 10 μl/min. Samples of antibody, prepared in HBS, were injected across the surface via the sample loop contained within the fluidics cartridge of the system, and dissociation was effected by injecting HBS only. The surface was regenerated by injection of 10 μl guanidinium hydrochloride (6 M, pH 6.0), a powerful regeneration method for small immobilized organic ligands and peptides, because only proteins are fully denatured, solubilized, and washed away during regeneration. Inhibition studies were carried out by co-incubation of the antibody with different haptens at a series of concentrations for 30 min at 10°C prior to injection. In the case of the anti-His-tag antibody, the inhibition studies were carried out with the surface carrying the His-peptide because, in contrast to a protein-containing surface, it can more easily be regenerated (6 M GdnCl, pH 6.0). The data analysis was carried out using the BIAevaluation software (Pharmacia) and Kaleidagraph (Synergy Software, U.S.A.).

RESULTS

ELISA and Competition ELISA

We first carried out an extensive epitope mapping study (Krebber *et al.*, unpublished) of the β-lactam binding antibody 2H10 (10) by ELISA and competition ELISA. The data obtained from competition ELISA (Fig. 1a) clearly show that the best antigen for the antibody 2H10 is benzoylampicillin, whereas the hydrolyzed form is bound less tightly (for formulas, see Fig. 1b). The major part of the recognition surface is the hydrophobic part of benzoylampicillin. This is also reflected by the recognition of benzoyl-DPG-OMe (for formula, see Fig. 1b), which is bound as tight as hydrolyzed benzoylampicillin, under the conditions of the competition ELISA (data not shown). Since the various competition ELISAs were not carried out according to Friguet *et al.* (13), we can only derive relative binding constants, but no absolute values. These competition ELISAs indicated clear differences between the different compounds, but it was not possible to detect differences between the antigens, when these were directly coupled to the solid phase. To rule out that the recognition of the maleimide linker is the reason for the leveling effects seen in standard ELISA, inhibition was also carried out with MBS–ampicillin coupled to DTT (for formula, see Fig. 1b). No difference in the inhibition between benzoylampicillin and DTT–MBS–ampicillin can be seen (data not shown). Therefore, the maleimide group is not a part of the epitope site of the antibody, consistent with a model of the structure of the 2H10 antibody (A. Lupas and A. Plückthun, unpublished).

Apparently, a leveling effect occurs due to the bivalency of the antibody at high hapten concentrations on the surface. A true ranking of the antigens and a determination of the tightest binding hapten was only possible in competition ELISA, a method governed by affinity in solution. It appears that avidity plays a major role in leveling the differences in the direct ELISA.

Solid-Phase BIAcore Studies

To obtain more quantitative data, three antigens (benzoylampicillin, hydrolyzed benzoylampicillin, and benzoyl-DPG-OMe, Fig. 1b) were chosen for determining their binding constants in the BIAcore instrument. The measurement was carried out following the usual direct determination of on- and off-rates, using antigen coupled to the surface as described above. The efficiency of coupling was tested by injecting a saturating amount of antibody 2H10 onto the derivatized surface. Signals of around 1000 RU were obtained and this is in a useful range for determining kinetic constants, following the standard method (7). Using a series of different concentrations of the antibody 2H10, kinetic data for the three haptens were calculated (Fig. 2), using the BIAevaluation software. From visual inspection, the dissociation phase looked curved. We calculated the expected k_{diss} values from the solution K_D values (see below) and k_{on} and obtained the steep dotted lines in Figs. 2b, 2d, and 2f (see below), which suggest that the kinetics are slowed down by rebinding and/or bivalency effects. The same standard analysis was carried out for the anti-His-tag antibody, where the saturating amount of antibody lead to R_{max} values of 1200 for the peptide surface and to 450 RUs for the His-tag protein surface (Fig. 3).

Because of the large differences obtained compared to the binding behavior seen in competitive ELISA (Fig. 1a), we wished to elucidate the cause for this discrepancy. We first describe in detail the method and assumptions for the standard binding constant determination (7). The association phase is described by the bimolecular reaction between the analyte at solution concentration (C) and the free binding sites on the surface ($R_{max} - R_t$), minus the simultaneously occuring dissociation:

$$\frac{dR_t}{dt} = k_{ass} \cdot C \cdot (R_{max} - R_t) - k_{diss} \cdot R_t, \quad [1]$$

more commonly written as

$$\frac{dR_t}{dt} = k_{ass} \cdot C \cdot R_{max} - (k_{ass} \cdot C + k_{diss}) \cdot R_t, \quad [2]$$

where k_{ass} is the rate constant for the association, k_{diss} is the rate constant for the dissociation, C is the concentration of analyte (in this case the antibody), R_t is the measured signal at time t, and R_{max} is the maximal response at saturating concentrations of analyte. When integrated, this yields

$$R_t = \frac{k_{ass} \cdot R_{max} \cdot C}{k_{ass} \cdot C + k_{diss}} \cdot (1 - e^{-(k_{ass} \cdot C + k_{diss}) \cdot t}), \quad [3]$$

which can directly be used for determining the observed rate constant $k_{obs} = k_{ass} \cdot C + k_{diss}$ and the initial slope $r_0 = k_{ass} \cdot C \cdot R_{max}$ as parameters by numerical fitting:

$$R_t = \frac{r_0}{k_{obs}} \cdot (1 - e^{-k_{obs} \cdot t}). \quad [4]$$

Plots of k_{obs} as a function of analyte concentration C were used to yield a new line with slope k_{ass} and intercept on the ordinate k_{diss}. The dissociation rates were evaluated from

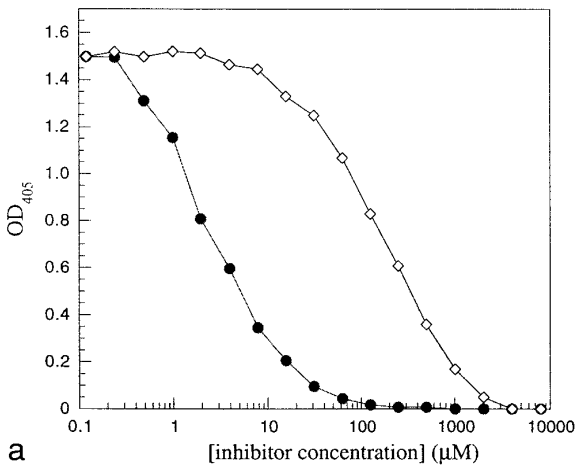
$$\frac{dR_t}{dt} = -k_{diss} \cdot R_t, \quad [5]$$

which upon integration with respect to time gives

$$\ln \frac{R_{t_1}}{R_{t_n}} = k_{diss} \cdot (t_n - t_1), \quad [6]$$

where R_{tn} is the response at time n and R_{t1} is the response at dissociation starting time 1. Theory predicts that a plot of R_{tn} versus time is a straight line.

While rarely stated explicitly, a number of tacit assumptions are made in this standard treatment. First and foremost, it is assumed that the measured processes are reaction limited, and dissociation is irreversible. Thus, rebinding of eluted antibody (monovalent or multivalent) will artificially slow down the off-rate. Moreover, this rebinding depends on available antigens on the surface and thus on the coating density as well as on the total amount of bound antibody. Consequently, plots of ln$R$ versus time are frequently nonlinear because with time, the surface empties and rebinding becomes more frequent. Even if they are nearly linear, as can be seen in the determination of the off-rate of the anti-His antibody (Fig. 3), the K_D value obtained from standard calculations (Eqs. [1] to [6]) using



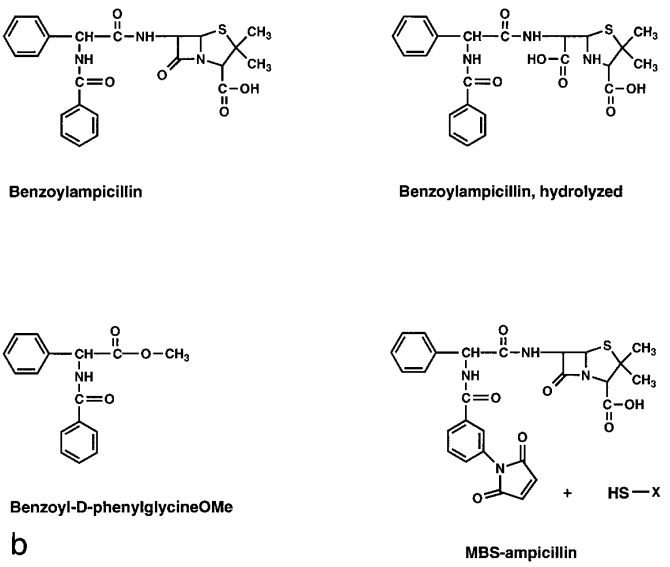


FIG. 1. (a) Competition ELISA: The surface-coated antigen was hydrolyzed benzoylampicillin coupled to reduced and denatured transferrin. $OD_{405\,nm}$ is plotted against the inhibitor concentration. The filled circles represent benzoylampicillin and the open diamonds denote hydrolyzed benzoylampicillin. Since the assay is not carried out with low surface coating (13), the inhibition curves can only be used to obtain relative binding constants. (b) Antigens used for the ELISA experiments and BIAcore kinetic analysis of the antibody 2H10. MBS–ampicillin was coupled via the double bond of the maleimide group to free SH groups (x, reduced transferrin or DTT or reduced cystamine).

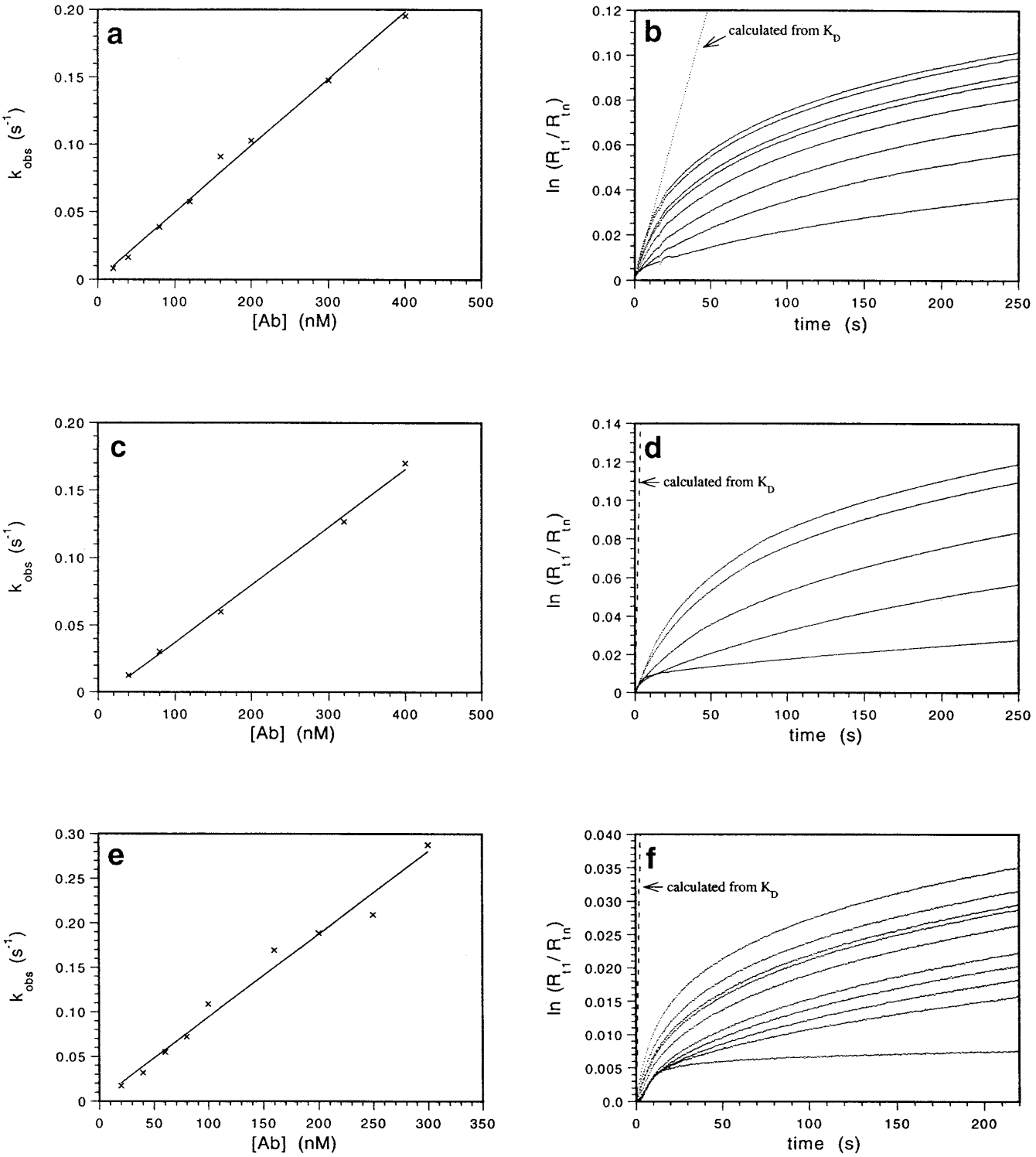


FIG. 2. On- and off-rate determination using the BIAevaluation software and Kaleidagraph. (a, c, and e) The k_{obs} values were plotted against the antibody concentration. k_{ass} can be calculated from the slope of the linear fit (Eq. [1]). (b, d, and f) ln (R_{t1}/R_{tn}) was plotted against the time of the dissociation phase to "determine" k_{diss}, as is commonly done, using the values from 50 to 200 s (Eq. [3]). The dotted lines were the expected dissociation curves calculated from K_D (obtained from Eq. [15]) and k_{ass}. a and b are benzoylampicillin ($k_{ass} = 5.5 \times 10^5$ M^{-1} s^{-1}; $k_{diss} = 2.7 \times 10^{-3}$ s^{-1}). c and d are hydrolyzed benzoylampicillin ($k_{ass} = 4.0 \times 10^5$ M^{-1} s^{-1}; $k_{diss} = 2.3 \times 10^{-3}$ s^{-1}). e and f are benzoyl–DPG–OMe ($k_{ass} = 7.0 \times 10^5$ M^{-1} s^{-1}; $k_{diss} = 1.4 \times 10^{-3}$ s^{-1}). Note the critical discussion of the k_{diss} values in the text.

surface on- and off-rates can still differ by a factor of 50 from the value obtained in free solution (see below). This indicates that all problems described in this paper also occur when R_{max} is in the recommended range (BIAcore manual) for determination of binding constants in BIAcore, and the problems may not be apparent from the shape of any plot.

Second, it is assumed that all binding events are governed by the same free energy of binding. This is a reasonable assumption, if all immobilized antigens are equally accessible and only monovalent complexes are involved. It is clearly not valid when bivalent binding may occur, since bridging complexes of different geometries will be obtained, leading to a *range* of on-rates and off-rates.

In addition to the above-mentioned problems, general concerns about measuring binding constants on surfaces remain. Precautions have to be taken to avoid artifacts from mass transport limitation (14). Furthermore, it has to be verified that the antibody has no affinity to the solid matrix itself, and that the coupled antigen is fully accessible and conformationally identical to the antigen of interest. The above-mentioned treatment (Eqs. [1] to [6]) is also not able to describe heterogeneous surfaces or conformational changes. Such systems can be analyzed by numeric integration (15, 16), but the method still relies on the biosensor reflecting microscopic rate constants. We show here examples where this is clearly not the case.

As can be seen in Fig. 2a for benzoylampicillin, the line obtained for k_{obs} versus antibody concentration is linear and the intercept with the ordinate gives a reasonable value (1.0×10^{-3} s^{-1}) for the dissociation rate constant. The dissociation rate is, however, not concentration-independent, as the plot in Fig. 2b shows. The same can be seen for the two other haptens (Figs. 2d and 2f). This can be due to a heterogeneity of off-rates (from the dissociation of monovalent and bivalent molecules). In addition, this is the typical effect for rebinding. Thus, at relatively low immobilization density (R_{max} = 1000) multivalent binding and reassociation take place for the β-lactam antibody–antigen system studied here, making the usual determination of the off-rates (Eqs. [5] and [6]) and thus the binding constants invalid. This nonlinearity cannot be due to the buffer change, since the resulting RU changes should be finished within 3–5 s, while the effects seen here last for more than 100 s. Lowering the amount of immobilized antigen from R_{max} = 5000 to $R_{max} \leqslant 1000$ had no effect on the apparent binding constants determined with this methodology. Injection of benzoylampicillin during the dissociation phase did lead to a faster dissociation rate (data not shown), a clear indication that rebinding of mono- and/or bivalent molecules is really a problem. We thought it would be instructive that the apparent K_D values still be determined by the standard methodology for comparison with more appropriate methods (see below) (Table 1).

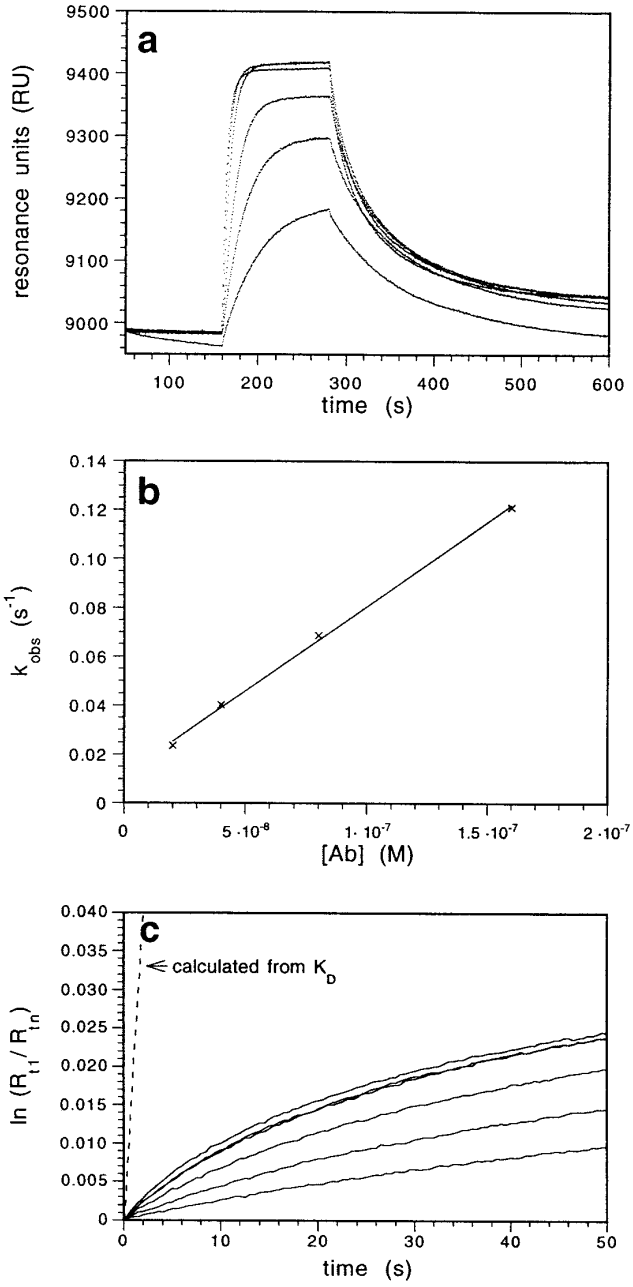


FIG. 3. (a) Overlay of the sensograms of the anti-His-tag antibody. The surface-coated antigen was the protein with a C-terminal His-tag. The antibody concentrations used were 20, 40, 80, 160, 240, and 320 nM. (b) The k_{obs} values from the sensograms of 20, 40, 80, and 160 nM were plotted against the antibody concentration to determine k_{ass} ($k_{ass} = 6.0 \times 10^5$ M^{-1} s^{-1}). (c) Analysis of the dissociation phase (280–330 s in a) ($k_{diss} = 6.0 \times 10^{-3}$ s^{-1}). Note the critical discussion of the k_{diss} values in the text.

These phenomena are, however, not only observed with bivalent antibodies: We also tested a scFv frag-

TABLE 1

Affinity measurements of the Anti-β-Lactam Antibody 2H10

Antigen[a]	K_D from kinetics[b] [M × 10^9]	K_D from inhibition (uncorrected)[c] [M × 10^8]	K_D from inhibition (corrected)[d] [M × 10^9]
Benzoylampicillin	5.0 ± 0.53	1.2 ± 0.34	4.8 ± 0.22
Hydrolyzed benzoylampicillin	5.9 ± 0.67	78 ± 1.1	340 ± 8.0
Benzoyl-D-phenylglycine–OMe	2.0 ± 0.64	330 ± 13	900 ± 6.0

[a] For formulas see Fig. 1.
[b] Calculated from k_{ass} and k_{diss} (Eqs. [1] and [6]).
[c] Not corrected for bivalency (Eq. [11]).
[d] Corrected for bivalency (Eq. [15]).

ment (Fig. 4), which was purified by affinity chromatography and afterward tested by size-exclusion chromatography. A symmetric, single peak at MW 27 kDa indicated that it is entirely monomeric (data not shown). This scFv also showed clear rebinding in the kinetic measurement of the BIAcore, indicated by its dramatic change in off-rate from near zero at the later stages after 500 s (BBS buffer phase in Fig. 4) to almost instantaneous elution by adding competitor (10 μM ampicillin and 100 μM ampicillin injection in Fig. 4). Therefore, the problem described before for the two whole antibodies is not limited to dimeric binding proteins, but also occurs for monomeric proteins. While we cannot formally rule out self-association of the scFv within the dextran matrix, we have no evidence for any aggregation tendencies of this antibody, and thus rebinding of monomeric molecules is the simplest explanation of the observed effects. This has now been demonstrated to occur with three unrelated antibodies and six different antigens.

Competition BIAcore

To overcome all these problems inherent in solid-phase measurement at once we worked out a method for measuring the binding constants in solution, using the BIAcore instrument for detection. The experiments not only give the K_D value for monovalent binding, but also some indication of the apparent values for multimeric binding (which do, by the very nature of the effect, also contain rebinding phenomena). Another advantage of this method is that direct comparisons of different haptens are possible with the same surface. For the competitive

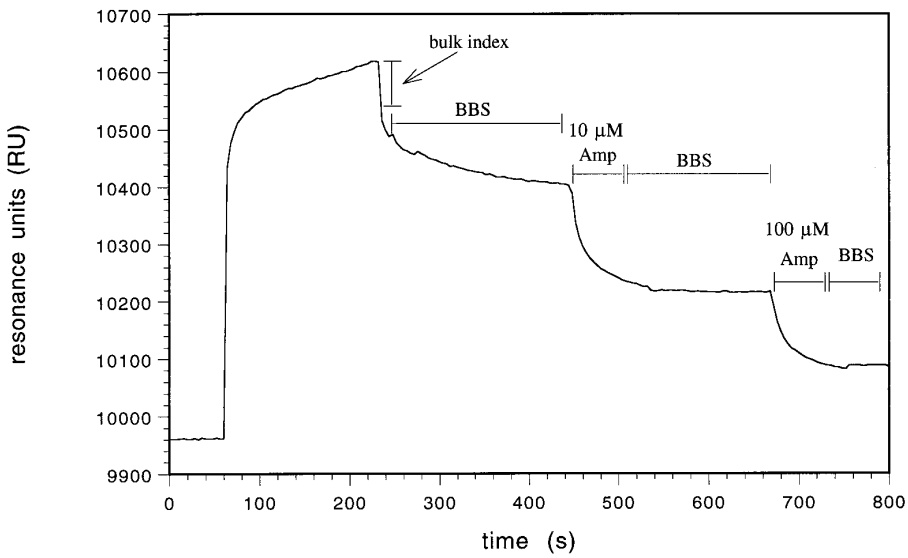


FIG. 4. Sensogram of the ampicillin-binding scFv fragment demonstrated to be monomeric. Ampicillin was coated to the surface and the purified scFv (89 nM) was injected, using 7 μl/min as flow rate. The bars indicate the different phases: 65–230 s, association phase; 230–232 s, buffer change (bulk index); 232–440 s, BBS buffer flow; 440–485 s, injection of 5 μl of 10 μM ampicillin; 485–670 s, BBS buffer flow; 670–715 s, injection of 5 μl of 100 μM ampicillin; 715–800 s, BBS buffer flow.

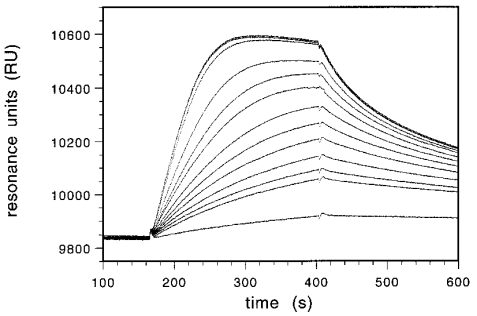


FIG. 5. Overlay of the sensograms from the inhibition experiment of the antibody 2H10 with benzoylampicillin. The concentration of the antibody was 80 nM. The concentrations of benzoylampicillin used in this study were 0, 5, 20, 30, 40, 50, 60, 70, 80, 90, 100, and 200 nM.

kinetic experiments, an antibody concentration close to the K_D (estimated from preliminary experiments or competition ELISA data) was chosen and kept constant in all experiments, while the concentration of the inhibitor was varied. In the present experiments the antibody concentrations were 50, 80, and 100 nM, which was necessary to obtain good signals.

We chose to study the same three antigens as for the solid-phase BIAcore. Hydrolyzed benzoylampicillin was chosen as the surface-coupled hapten for practical reasons, because no hydrolysis of the β-lactam ring can occur and thus the surface will remain unchanged over time. All experiments could be carried out in only one flow cell. From initial estimates of K_D from inhibition ELISAs, a series of runs was performed in which the concentrations of the analyte were chosen around the K_D value estimated in the first run. After preincubation, the equilibrium mixture was injected onto the surface and analyzed for free antibody. The on-rate is always proportional to the concentration of antibody with free binding sites. If the equilibrium is not displaced by antibody being captured from solution by binding to the surface, and if the off-rate is negligible (see Discussion), k_{obs} ($=k_{ass}\cdot C + k_{diss}$) can be used directly as a readout of the equilibrium binding.

A typical sensogram of the inhibition series is given in Fig. 5 for benzoylampicillin. The calculations of the k_{obs} values from the time courses, needed for the K_I determination, were performed with the BIAevaluation software (Eqs. [1] to [4]). We first describe the situation for a monovalent antibody fragment. The K_I values of the different haptens were calculated using the equation

$$k_{obs} = k_{obs^0}\cdot\frac{[Ab_{free}]}{[Ab_{tot}]}, \qquad [7]$$

which is equivalent to

$$k_{obs} = k_{obs^0}\cdot\frac{1}{[Ab_{tot}]}\cdot([Ab_{tot}] - [Ab\cdot I]), \qquad [8]$$

where $[Ab\cdot I]$ is the antibody–inhibitor complex. Since K_I is defined by

$$K_I = \frac{[Ab_{free}]\cdot[I_{free}]}{[Ab\cdot I]}, \qquad [9]$$

which is equivalent to

$$K_I = \frac{([Ab_{tot}] - [Ab\cdot I])\cdot([I_{tot}] - [Ab\cdot I])}{[Ab\cdot I]}, \qquad [10]$$

$[Ab\cdot I]$ can be expressed as a function of K_I, Ab_{tot}, and I_{tot} from solving Eq. [10] for $[Ab\cdot I]$ and inserting it into Eq. [8]:

$$k_{obs} = k_{obs^0}\cdot\frac{1}{[Ab_{tot}]}\cdot\left([Ab_{tot}] - \left(\frac{[Ab_{tot}] + [I_{tot}] + K_I}{2}\right) + \sqrt{\left(\frac{[Ab_{tot}] + [I_{tot}] + K_I}{2}\right)^2 - [I_{tot}]\cdot[Ab_{tot}]}\right), \qquad [11]$$

where k_{obs^0} is k_{obs} of the noninhibited sample, k_{obs} is the value at inhibitor concentration I_{tot}, Ab_{tot} is the total antibody concentration, I_{tot} is the inhibitor concentration, and K_I is the inhibition constant to be determined. This equation can be directly fitted to the experimental data, with K_I as the sole parameter.

For an antibody with two binding sites, the K_I values have to be corrected. Because the antibody could have bound zero, one, or two antigens, the observed binding rate is no longer simply proportional to free antibody combining sites. This problem can be solved using the same strategy described previously by Stevens (17) for an ELISA experiment. Defining f as the fraction of bound combining sites, f can be interpreted as the probability that a randomly selected binding site is occupied and $1 - f$ as the probability that the site is free. Double liganded antibody occurs with the frequency f^2, and all other species together then with the frequency $1 - f^2$. All the other species except the doubly liganded antibody are assumed to bind to the surface, and the observed rates, called k_{obs-bi}, depend on the concentration of antibodies with at least one site free.

$$k_{obs-bi} = (1 - f^2)\cdot k_{obs^0}. \qquad [12]$$

In the monovalent case, $k_{obs} = (1 - f)\cdot k_{obs^0}$, which is identical to Eq. [8]. The concentration of free binding sites $1 - f$ is then

$$1 - f = 1 - \sqrt{1 - \frac{k_{obs-bi}}{k_{obs^0}}}. \qquad [13]$$

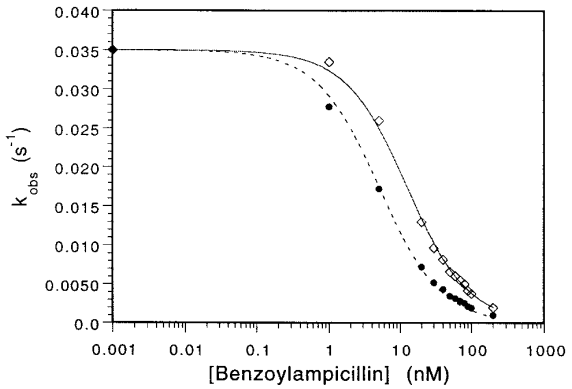


FIG. 6. The k_{obs} values from Fig. 5 (open diamonds) were plotted against the inhibitor concentration (benzoylampicillin) and fitted by Eq. [11] (ignoring bivalency). The filled circles are the k_{obs} values corrected for bivalency by Eq. [14] and then fitted by Eq. [11].

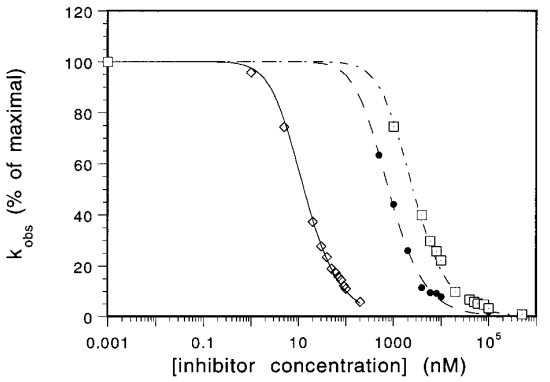


FIG. 7. The k_{obs} values obtained from the time courses were plotted against the inhibitor concentration and directly fitted by Eq. [15]. The inhibitors benzoylampicillin (◇), hydrolyzed benzoylampicillin (●), and benzoyl–D-phenylglycine–OMe (□) are shown.

The values obtained in an experiment with bivalent antibody, k_{obs-bi}, thus need to be corrected by

$$k_{obs} = \left(1 - \sqrt{1 - \frac{k_{obs-bi}}{k_{obs}^0}}\right) \cdot k_{obs}^0 \qquad [14]$$

for use in Eq. [11]. In Fig. 6, a comparison of the directly fitted k_{obs} values (ignoring the bivalency of the antibody) with the corrected k_{obs} values (using Eq. [14] and then Eq. [11] to account for bivalency) is shown. Alternatively, the k_{obs-bi} values can be directly fitted by Eq. [15].

$$k_{obs-bi} = k_{obs}^0 \cdot \left[1 - \frac{1}{4 \cdot [Ab_{tot}]^2} \cdot \left(\left(\frac{2 \cdot [Ab_{tot}] + [I_{tot}] + K_I}{2}\right)\right.\right.$$

$$\left.\left. - \sqrt{\left(\frac{2 \cdot [Ab_{tot}] + [I_{tot}] + K_I}{2}\right)^2 - [I_{tot}] \cdot 2 \cdot [Ab_{tot}]}\right)^2\right] \qquad [15]$$

In Fig. 7, the three K_I values for the three different haptens were directly determined and fitted by Eq. [15].

The second antibody system tested in this study was directed against the his-tag, an oligohistine tail used in protein purification (18). Two inhibition measurements were carried out, one using the peptide as a competitive ligand for determining its binding constant, the other one to determine the K_I of imidazole. In Table 2, a comparison of the K_D for the peptide obtained from determining on- and off-rates (Eqs. [1] to [6]) and from inhibition in solution (Eq. [15]) is shown. The K_D values were corrected for bivalency by use of Eq. [15]. Also in this case, the differences between the data from on- and off-rates and those from solution inhibition are very dramatic.

To further substantiate that the value determined by inhibition BIAcore is the correct thermodynamic binding constant, we also created the scFv of the anti-His-tag antibody. The scFv showed the plateau behavior typical for fast on- and off-rates, and thus it was not possible to obtain kinetic data with the affinity-purified scFv fragment. The plateau data, however, indicate that the K_D for the His-peptide is indeed around 10^{-6} M. The K_I of imidazole was determined to be 4×10^{-4} M by competition BIAcore, which would be impossible to determine from binding kinetics.

DISCUSSION

In this paper we demonstrate that the standard determination of binding constants from on- and off-rates may not reproduce binding constants in solution, and not even relative affinities. Since this is due to the biosensor response time curve no longer reflecting the true dissociation event, but continuous rebinding of unknown magnitude (see the effect of adding competitor in Fig. 4), this cannot be rectified by a more sophisticated data treatment such as direct numerical integration (15, 16). To do this we would need information on the rebinding probability of the antibody, which we cannot obtain.

TABLE 2

Affinity Measurements of the Anti-His-Tag Antibody

Constants	Ligand	Value [M × 10^8]
K_D from kinetics[a]	His-tag protein	1.0 ± 0.19
K_D from kinetics[a]	His-peptide	0.2 ± 0.016
K_D from inhibition[b]	His-peptide	40 ± 3.5
K_D from inhibition[b]	Imidazole	40,000 ± 4500

[a] Obtained from k_{ass} and k_{diss} (Eqs. [1] and [6]).
[b] Obtained from Eq. [15].

We show that competition BIAcore is a very powerful tool for affinity ranking of molecules over a wide range of binding constants (e.g., in epitope mapping or receptor binding assays) and for determination of mono- and multivalent binding constants in only one experiment. Karlsson (9) has recently described a similar approach, although no comparative data between solution binding and surface binding were provided, and only a single compound was tested. He also used a slightly different strategy, which we wish to compare now.

The assumption underlying our treatment here is that k_{obs} (the rate constant describing the observed association phase) is proportional to Ab_{free} (or the quantity $1 - f^2$ as defined above), and thus a measure of the equilibrium binding of an inhibitor. The first condition for this being valid is that $k_{on} \approx k_{obs}$, which is the case if k_{diss} is negligible (Eq. [1]). This assumption was practically always found to be valid (from evaluating k_{obs} and k_{diss}) whenever surface dissociation was sufficiently slow to obtain kinetic measurements. Since only *surface* dissociation is relevant, a slow off-rate can even be achieved via bivalency and rebinding.

At the highest inhibitor concentrations, Ab_{free} will become very small, and the assumption may be no longer valid. However, the influence of these points on the K_I value calculated from Eq. [11] or Eq. [15] was found to be very small, and this simplification is thus still permissible.

The second condition is that the surface-bound antigen does not disturb the preestablished equilibrium in solution by trapping the antibodies. This is very similar to the condition of the ELISA according to Friguet *et al.* (13). Since the BIAcore instrument uses a flow cell, this condition can be rephrased: Only insignificant dissociation of the complex is allowed to occur during the time of contact of the complex with the chip surface. The surface has dimensions of 2 mm · 500 μm · 50 μm (60-nl volume), and at a flow rate of 10 μl/min, the contact time is less than 0.5 s. [This ignores the parabolic velocity profile in the cell (14), but probably describes the bulk analyte in a reasonable way]. If we allow an error of 1% (i.e., 1% complex dissociation within 0.5 s) we can calculate the maximal permissible off-rate to fulfill this condition:

$$[Ab \cdot Ag_t] = [Ab \cdot Ag_0] \cdot e^{-k_{diss} \cdot t}, \qquad [16]$$

where $Ab \cdot Ag_t$ is the complex present at time t, and $Ab \cdot Ag_0$ at time 0. Allowing 1% dissociation in 0.5 s we obtain

$$0.99 = e^{-k_{diss} \cdot \Delta t}. \qquad [17]$$

If $\Delta t = 0.5$ s, the maximal permissible k_{diss} is 2×10^{-2} s^{-1}. If 10% error in the final absolute binding constants is permissible (which is probably already the case from inaccuracies in solution concentrations), k_{diss} may be as large as 2×10^{-1} s^{-1}.

The third condition to be met is that k_{obs} is proportional to Ab_{free}. Assuming that k_{diss} is negligible under the conditions of the experiment (see above), this is equivalent to k_{obs} being proportional to Ab_{tot} in the absence of inhibitor. Note that this assumption is inherent in *all* BIAcore measurements for monovalent molecules. It will not necessarily be true, however, if complicated kinetic behavior in association phases is seen, perhaps when monovalent and multivalent binding are occuring concurrently. Proportionality was found to be the case in all the experiments here. However, if such problems are observed, one may increase the surface concentration of the immobilized ligand to the point that mass transport is clearly rate limiting (since the association process is then so fast that it is no longer rate determining) (9).

In conclusion, k_{obs} is a correct measure of Ab_{free} under the conditions of the experiments described here, since all three assumptions are valid. It is also not required to use initial rates (9), since at very fast rates these are more prone to contain artifacts from buffer mixing. Furthermore, if mass transport is not *clearly* limiting, curved on-rates may still result, which are difficult to fit to the expected straight line and would therefore require a series of experiments under various conditions to fit a more sophisticated model (14).

Other precise methods to determine binding constants in solution are known (19). The most generally applicable is the method of Friguet *et al.* (13), which is in essence an inhibition ELISA, in which care is taken that the solid-phase ligand does not displace the equilibrium. This usually requires a series of experiments to optimize the coating density. The competition BIAcore system described here is more versatile, since it does not need any other method to detect the bound species and is therefore independent from having detection antibodies or any labels. While the ELISA according to Friguet *et al.* (13) uses simple equipment, more information is obtained from the competition BIAcore experiments, since some kinetic data are obtained as well.

There are several advantages to the competition BIAcore method: First, only one surface is needed to carry out the whole experiment. Second, the direct comparison of affinities is possible. Third, if a protein antigen is denatured on the surface, a measurement with native protein in solution will still be possible, if the antibody also binds to the denatured protein on the matrix. The affinity ranking and K_I determination would not be disturbed, because the antigens in solution would be native. Fourth, this approach may also be useful for protein or peptidic antigens and in fact for any interacting compound, whenever avidity or sig-

nificant rebinding cannot be completely ruled out. Fifth, this approach might also allow the broadening of the affinity window of BIAcore, which is now limited from about 10^{-5} to 10^{-10} M. With the new approach, only free antibody concentration is measured, and therefore dissociation constants of high-affinity antibodies with K_D significantly lower than 10^{-10} M should be possible to determine, limited only by the sensitivity of the instrument. High K_D values (weak binding), impossible to be determined in normal BIAcore procedure, are also within the range of the new approach. Such an example has been provided with the binding constant of imidazole to the anti-His-tag antibody (4×10^{-4} M), which is the lowest affinity precisely determined in BIAcore of which we are aware.

Why are the differences between surface affinities and solution affinities so large? We believe that the problem may be especially pronounced with small molecules which have a low steric requirement and thus fast on-rates and are easy for the receptor to capture productively on the surface, leading to very efficient rebinding and, if applicable, bivalent binding. Many proteins will simply be too big to allow an antibody to bind bivalently. Furthermore, haptens generally tend to have faster on-rates and thus, in the dissociation phase, are much more likely to recapture the antibody to the surface, before the bulk flow transports it out of the flow cell.

Rebinding plays a significant role as indicated from the concentration dependence of the off-rates. Not in all experiments did the inclusion of the hapten into the buffer in the elution phase increase the off-rate to the value expected from the affinity measurements. Possibly the effective concentration of the surface-bound hapten is so high that soluble hapten can only partially compete with it.

A most interesting (and worrisome) finding is that not even the relative affinities determined by surface measurements reflect the true binding constants. Rather, there seems to be a "leveling off" at highest affinities. It is possible that the higher affinities lead to very slow off-rates so that over the time window studied here, only the monovalent antibodies dissociate, while the bivalent ones are not taken into account, thus applying in effect different "weights" to the monovalent and bivalent off-rates, dependent on their absolute value. It appears that not in all published data on using kinetic measurements for deriving affinities, was enough care taken to exclude such effects of rebinding and bivalency. From the analysis presented here, we predict the problems to be most pronounced for analytes with fast on-rates. These findings have profound implications in using BIAcore to study affinity maturation of antibodies *in vivo* and in phage libraries.

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