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Compensation for Loss of Ligand Activity in Surface Plasmon Resonance Experiments

Raimund J. Ober*,† and E. Sally Ward†,‡,[1]

*Center for Systems, Communications and Signal Processing, Eric Jonsson School of Electrical Engineering and Computer Science, University of Texas at Dallas, Richardson, Texas 75083-0688; and †Cancer Immunobiology Center NB9.106 and ‡Center for Immunology, University of Texas Southwestern Medical Center, 6000 Harry Hines Boulevard, Dallas, Texas 75390-8576

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The determination of equilibrium binding constants is an important aspect of the analysis of protein–protein interactions. In recent years surface plasmon resonance experiments (e.g., with a BIAcore instrument) have provided a valuable experimental approach to determining such constants. The standard method is based on measuring amounts of analyte bound at equilibrium for different analyte concentrations. During the course of a typical surface plasmon resonance experiment the measured equilibrium levels for a given analyte concentration often decrease. This appears to be due to a loss of activity of the protein coupled to the sensor chip or other phenomena. The loss in signal can lead to an erroneous determination of the equilibrium constant. A data analysis approach is introduced that aims to compensate for the loss of activity so that its influence on the results of the experiments is reduced.

Key Words: **loss of activity; surface plasmon resonance; BIAcore; equilibrium analysis.**

The determination of the equilibrium binding constant of protein–protein interactions is an important application of the use of a biosensor such as the BIAcore instrument (see, e.g., (1) and references therein). The use of optical biosensors for the analysis of macromolecular interactions offers several advantages over more conventional approaches to affinity determination. First, the interacting components need not be labeled. Second, the amounts of material needed for analysis are relatively small. Third, multiple potential interactions can be analyzed in a relatively short time period. In a BIAcore instrument soluble analyte is flowed over immobilized ligand and binding is monitored in real time using surface plasmon resonance (SPR)[2] technology.

For the determination of the equilibrium binding constant of the interaction, equilibrium binding values (R_{eq}) are measured for various analyte concentrations. This means that a series of injections with differing analyte concentrations is carried out, each injection producing a sensorgram that yields a (dynamic) equilibrium value for the interaction that is being studied. The equilibrium constant is then typically determined using a Scatchard-type analysis. Here we propose a method to deal with a problem that is often observed in BIAcore experiments. Over time the binding activity of the immobilized ligand on the chip appears to decrease. This can be for a variety of reasons such as protein denaturation. The result of such degeneration within one experiment is that equilibrium values that are measured later in the series of sensorgrams have lower values than they would if no degeneration had occurred. This means, for example, that an otherwise linear Scatchard plot may no longer be linear. Another resulting problem may be that an erroneous equilibrium constant is determined.

The purpose of our study is to demonstrate that such problems can occur in practical situations and that they may indeed result in an erroneous determination of the equilibrium constant and to propose an approach to diagnose and reduce the impact of these problems. Diagnosing the potential problem is clearly of importance and it is doubtful that current experimental de-

[1] To whom correspondence should be addressed. E-mail: Sally.Ward@UTSouthwestern.edu.

[2] Abbreviations used: SPR, surface plasmon resonance; HEL, hen egg lysozyme; RU, resonance units; NTA, nitrilotriacetic acid; MBP, myelin basic protein; MHC, major histocompatibility complex; PBS, phosphate-buffered saline.

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signs allow this to be done effectively. If a problem is observed the question arises as to how to remedy it. There are fundamentally two ways of doing this. One is to change the experiment so that loss of activity no longer occurs. This is certainly the most desirable approach if sufficient time and material are available for potentially several repeat experiments until the underlying cause of the problem is determined and removed. Since BIAcore is typically used to provide relatively fast results without a significant loss of material we developed an alternative data analysis approach in which the data are compensated for the loss of activity of the ligand. The main advantage of this method is that typically no additional data need to be acquired provided that the experiment has been set up using the proposed experimental design.

As the main experimental system for this study we chose the hen egg lysozyme–D1.3 Fab (HEL–D1.3) interaction as the D1.3 antibody–HEL and D1.3 Fv–HEL interactions have been extensively studied (2–6). As a result, the fundamental aspects of the interaction such as equilibrium constant (using fluorescence-based and SPR methods) and stoichiometry have all been carefully examined by us and others.

MATERIALS AND METHODS

Theory

The standard equation that describes the result of a measurement in a flow cell for a 1:1 interaction is given by (see, e.g., (7))

$$\frac{R(t)}{dt} = k_{on}C(t)(R_{max} - R(t)) - k_{off}\,R(t), \qquad [1]$$

where $R(t)$ is the measured signal in resonance units (RU), k_{on} is the association rate constant, k_{off} is the dissociation rate constant of the interaction, R_{max} is the maximum analyte binding capacity in RU, and $C(t)$ is the concentration of the analyte that is flowed over the chip.

If during an association phase the constant concentration C_k is applied the limit signal, which we call the *equilibrium signal* R_{eq}, is given by (see, e.g., (7))

$$R_{eq}(C_k) = \frac{1}{K_d + C_k}\,C_k R_{max}, \qquad [2]$$

where $K_d := k_{off}/k_{on}$ is the *equilibrium dissociation constant.*

The standard way to obtain the equilibrium dissociation constant is to perform a number of experiments with different levels of concentration, $C_1, C_2, \ldots, C_n$, for the analyte. For the corresponding equilibrium values $R_{eq}(C_1), R_{eq}(C_2), \ldots, R_{eq}(C_n)$ we have the relationship (see, e.g., (7))

$$\frac{1}{C_k}\,R_{eq}(C_k) = -\frac{1}{K_d}\,R_{eq}(C_k) + \frac{1}{K_d}\,R_{max},\ 1 \le k \le n. \qquad [3]$$

Use of a Scatchard-type plot of $R_{eq}(C_k)/C_k$ versus $R_{eq}(C_k)$ allows for the estimation of K_d by estimating the slope of the linear graph.

To address the problem of decaying sensitivity of the chip we propose to model the maximum analyte binding capacity of the chip in Eqs. [3] and [1] to be a decaying function of time, e.g.,

$$R_{max}(t) = \beta e^{-\alpha t} + \gamma,\ t \ge 0, \qquad [4]$$

for some constants $\alpha \ge 0$, $\beta \ge 0$, and $\gamma \ge 0$. But we assume that the decay process is sufficiently slow so that it can be adequately approximated by a time constant parameter R_{max} in Eq. [1] to describe the response to an analyte injection. The time parameter indicates the midpoint of the time interval at which the equilibrium value was attained, relative to the starting time of the full experiment. For the time measurement all experiments are taken into consideration. With the time adjustment for R_{max}, Eq. [2] for the equilibrium value R_{eq} is therefore also time dependent and needs to be rewritten as

$$R_{eq}(t_n, C_k) = \frac{1}{K_d + C_k}\,C_k R_{max}(t_n) = \frac{1}{K_d + C_k}\,C_k(\beta e^{-\alpha t_n} + \gamma), \qquad [5]$$

$t_1 < t_2 < \cdots < t_n < \cdots < t_N$, where t_n is the time point corresponding to the equilibrium value for the nth experiment/injection and N is the total number of equilibrium values that make up the experiment.

Our proposed approach is to acquire estimates for several concentrations C_k and to then simultaneously fit the above Eq. [5] to the resulting data. We therefore obtain estimates for K_d, α, β, and γ. Of those of course only the estimate for K_d is typically of interest.

Other models for the decay may be more suitable than the simple exponential decay model in Eq. [4] to describe the loss of activity of the interaction. In fact for one of the interactions that were studied here we found that the decay model

$$R_{max}(t) = \beta_1 e^{-\alpha_1 t} + \beta_2 e^{-\alpha_2 t} \qquad [6]$$

better described the data.

The optimization routine was implemented in Matlab making use of a gradient-based (Levenberg–Marquardt) optimization routine in the Optimization Toolbox (8).

Source of Proteins

Hen egg lysozyme was purchased from Sigma. D1.3 Fab fragments were purified from recombinant *Escherichia coli* cells transformed with a previously described expression plasmid (2, 9). This plasmid was modified by the insertion of a polyhistidine tag at the 3′ end of the $C\kappa$ domain using designed oligonucleotides and standard methods of molecular biology. Recombinant protein was expressed and purified using Ni^{2+}–NTA-agarose as described (10). Recombinant complexes comprising the N-terminal 11 amino acids of myelin basic protein (MBP) covalently tethered to the MHC class II protein I-A^u (MBP1-11[4Y]:I-A^u) were expressed in baculovirus-infected insect cells and purified as described in (11). The MBP epitope has a position 4 substitution of tyrosine to stabilize the complexes (11). A hybridoma secreting the anti-I-A^u antibody 10.2.16 (12) was obtained from the ATCC. The 10.2.16 antibody was purified using protein G–Sepharose and Fab fragments generated by papain digestion.

Surface Plasmon Resonance Experiments

All experiments were run using a BIAcore 2000 instrument. Flow cells of sensor chips (CM5 or C1) were coupled with D1.3 Fab fragment or biotinylated MBP1-11[4Y]:I-A^u complexes at densities ranging from 280 to 1500 RU. MBP1-11[4Y]:I-A^u complexes were coupled in oriented fashion by first coupling flow cells of CM5 chips with streptavidin via amine coupling. In contrast, D1.3 Fab was coupled directly via amine coupling. Flow cells coupled with coupling buffer only (for D1.3 Fab fragments) or with streptavidin only (for MBP1-11[4Y]:I-A^u complexes) were used as reference cells in the experiments. HEL (for D1.3 Fab) or 10.2.16 Fab (for MBP1-11[4Y]:I-A^u) fragments were injected over the flow cells at concentrations ranging from 5 to 100 nM (HEL) or 33 to 1000 nM (10.2.16 Fab) in PBS, pH 7.2, plus 0.01% Tween 20 and 0.02% sodium azide. Injection times were 24 min for HEL and 12 min for 10.2.16 Fab fragment. The injection times were chosen to ensure that during the association phase all sensorgrams reached the equilibrium level. PBS, pH 7.2, containing 0.01% w/v Tween 20 and 0.02% w/v sodium azide was used as running buffer for all experiments and analyte injections were carried out using the kinject command. Buffers were degassed and filtered through 0.2-μm cut-off filters prior to use. All experiments were carried out using a flow rate of 10 μl/min. In some experiments with D1.3 Fab a strip was included that comprises a 20-μl injection (2 min) of 0.1 M NaCl and 0.1 M glycine (pH 3). Experiments were run using programmed methods and in all cases complete dissociation of analyte to a steady-state level was observed prior to the subsequent injection.

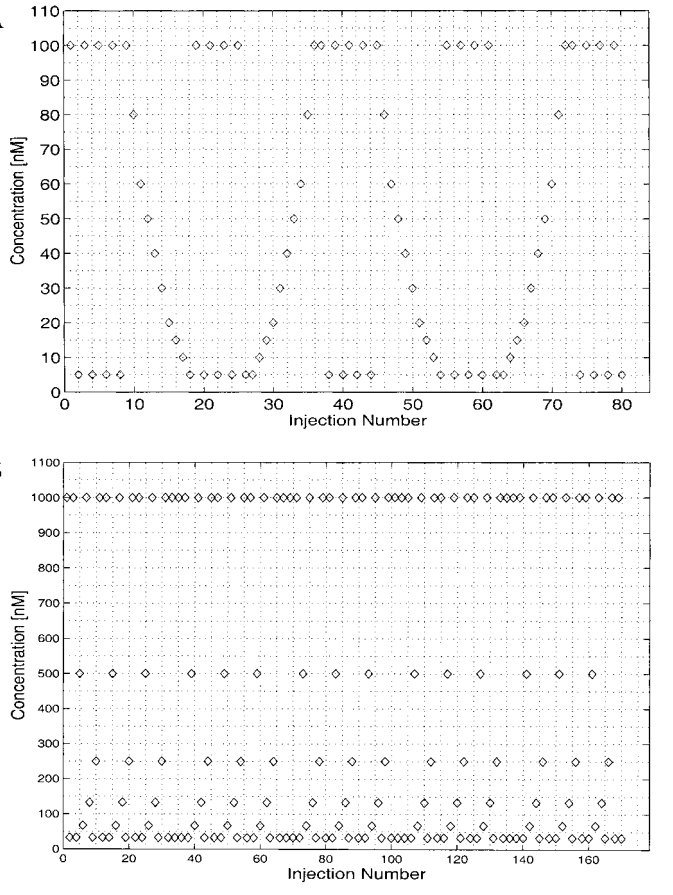

FIG. 1. A graphical representation of the injection levels for the experiments discussed under HEL–D1.3 Fab interaction (A) and I-A^u:10.2.16 Fab interaction (B). The *x* axis of the plots shows the number of the injection, whereas the *y* axis shows the concentration of analyte that is being injected. The concentration levels of HEL used were (A) 5, 10, 15, 20, 30, 40, 50, 60, 80, and 100 nM and of 10.2.16 Fab were (B) 33, 67, 133, 250, 500, and 1000 nM.

Data Processing

Before the data were analyzed they were zero-adjusted and the reference cell signal was subtracted. Sensorgrams that exhibited instrumental artifacts were excluded from the analysis. The equilibrium value of an association sensorgram that has reached equilibrium was calculated by typically averaging 30 data points.

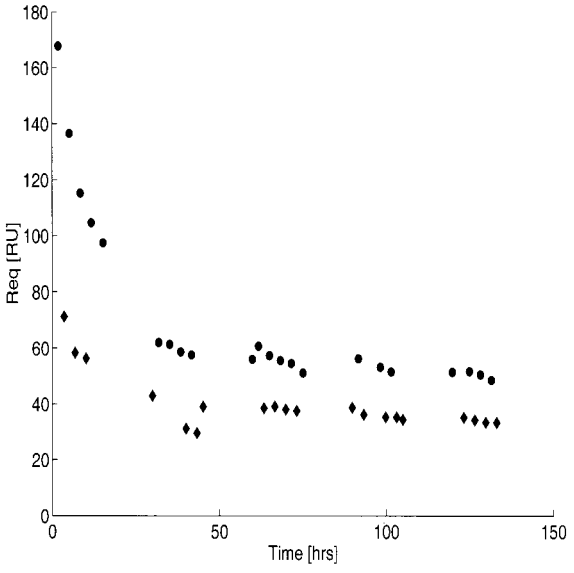

FIG. 2. Measured equilibrium values as a function of time for the experiment (see text for more details) in which a CM5 chip was coupled with D1.3 Fab and HEL was flowed across the chip with flow rate 10 μl/min. The depicted measurements were obtained in flow cell 3 for injection concentrations of 5 nM (◆) and 100 nM (●). The decrease in measured equilibrium values for these repeat injections is clearly visible.

RESULTS

Investigation of Loss of Activity

HEL–D1.3 Fab interaction. To investigate the impact of loss of activity on the determination of the equilibrium constant through Scatchard analysis we chose the very well characterized hen egg lysozyme–D1.3 antibody system (2–6). In the current study Fab fragments were used in preference to Fvs as the latter suffer instability due to VH–VL dissociation (13). We chose to couple the D1.3 Fab to the sensor chip rather than HEL as it is significantly larger, making steric hindrance of the binding site less likely to occur due to the random amine coupling. Fab fragments were used in preference to complete antibody as we aimed for a random distribution of binding sites on the chip.

A flow cell of a CM5 chip was coupled with D1.3 Fab fragment using amine coupling. A series of injections was carried out to obtain equilibrium values for the determination of the equilibrium dissociation constant. A graphical description of the sequence of analyte injections is given in Fig. 1.

In the experiments that were carried out loss of activity can be clearly detected (see Fig. 2 for a presentation of some of the results). This is particularly so in the early parts of each experiment. In the presented data it can be seen that the measured equilibrium values decrease significantly for repeat experiments. This phenomenon was observed for several other independent experiments that were carried out with ligand coupled at various densities on different flow cells (data not shown).

The observed decrease of measured equilibrium values for the same concentration of analyte is attributed to loss of activity of the ligand. To exclude the possibility that loss of activity of the analyte is responsible for the observed decrease in signal level, injections were repeated with fresh analyte (stored at either 4 or −20°C) at the end of each experiment. The resulting equilibrium values showed that the observed loss of signal level is not due to degeneration of the analyte.

The ultimate aim of this study is not to examine in fine detail the reasons for the loss of activity of the interaction. Nevertheless we wanted to exclude the possibility that basic experimental conditions are responsible for the effect. A question that naturally arises is whether “stripping” of the chip with acid and or alkali, for example, causes the effect. The above-discussed experiment (see Figs. 2, 3, and 5) was carried out including acid (pH 3) stripping of the chip after dissociation. An analogous experiment was also carried out on a new CM5 chip without stripping the chip. Analogous to the case when stripping was used, loss of activity is also observed if the experiment is performed without stripping (Fig. 6). In fact, using our proposed data analysis method (see below) both experiments led to similar equilibrium dissociation constants (K_d = 4.2 nM for the experiment with stripping and K_d = 3.8 nM for the experiment without stripping).

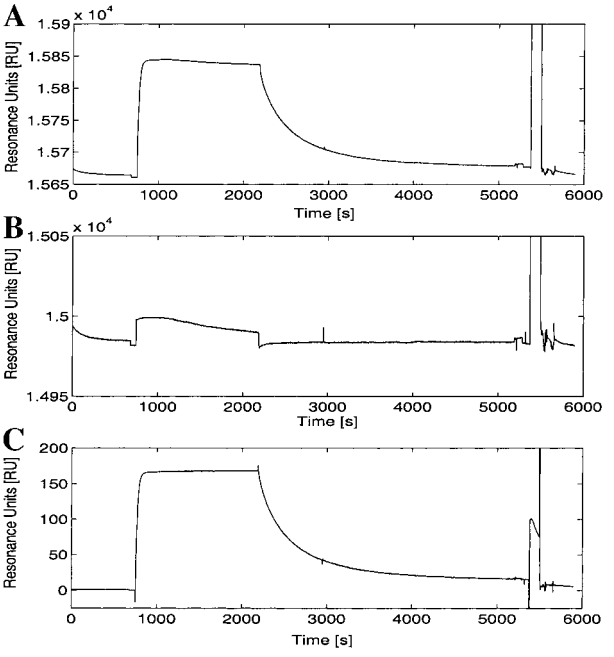

FIG. 3. Sensorgram of one of the 100 nM injections for the D1.3 Fab–HEL experiments (see text) including stripping signal. (A) Measured sensorgram. (B) Reference cell sensorgram. (C) Sensorgram after reference cell subtraction.

To investigate whether the dextran on the CM5 chip used in the above experiment was responsible for the observed loss of activity, a similar experiment was carried out using the C1 chip in which the analyte is not coupled to dextran but rather is coupled via amine coupling to carboxyl groups that are attached directly to the surface layer of the chip. In contrast, the CM5 chip has a layer of dextran and the ligand is coupled to carboxyl groups on the dextran. Also with this surface we observed a loss of activity phenomenon similar to the one with the CM5 chip (data not shown).

I-A^u:10.2.16 Fab interaction. To demonstrate that the observed loss of activity is not specific to the HEL–D1.3 system we also investigated a further interaction. A flow cell of a CM5 chip coupled with streptavidin was subsequently loaded with biotinylated MHC class II protein I-A^u bound to an MBP peptide. Injections of 10.2.16 Fab were carried out with different concentrations in a sequence as depicted in Fig. 1. The layout of the experiment is arranged in blocks which can be repeated until sufficient data are acquired to reliably estimate the equilibrium constant. The block of injections is started and finished by a number of injections of the highest and lowest concentration levels so that sufficient data are available to estimate the parameters that describe the loss of activity of the ligand. Loss of activity was also observed for this interaction (see Fig. 7). Since the I-A^u molecule is site specifically labeled the experiment also shows that the amine coupling in the HEL–D1.3 Fab interaction is not the prime cause for loss of ligand activity. Fab fragments were used in preference to complete antibodies as analyte to avoid valency effects.

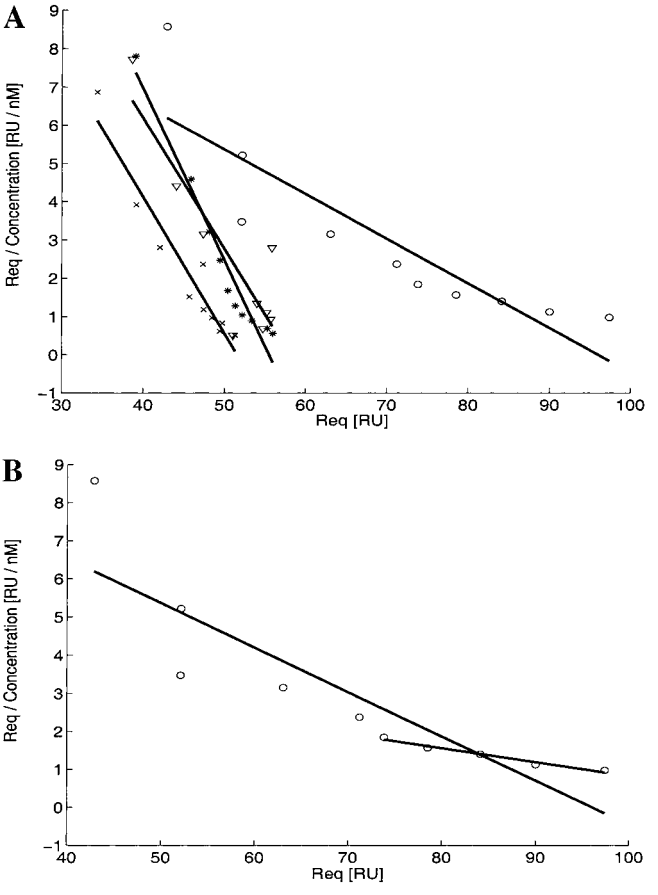

FIG. 4. (A) Scatchard type analyses are shown for four subsets of data points acquired as part of the experiment described under HEL–D1.3 Fab interaction (Figs. 1A and 2). The estimated equilibrium dissociation constants for each of the data subsets differ significantly. The first set of data points (Data set 1 (○)) was obtained as a result of a decreasing set of analyte injections starting at 100 nM and ending with 5 nM. The second set of data points (Data set 2 (*)) of equilibrium values was the result of an increasing set of injections starting at 5 nM and ending at 100 nM. The third set of data points (Data set 3 (▽) is a later repeat of the injections for Data set 1, and similarly Data set 4 (×) is the result of a repeat of the experiments that led to Data set 2. The estimated equilibrium dissociation constants are Data set 1, 8.6 nM; Data set 2, 2.2 nM; Data set 3, 2.9 nM; Data set 4, 2.8 nM. (B) Data set 1 is shown with a Scatchard type fit for the complete data set and a fit for the first five data points of the experiment. An equilibrium dissociation constant determination based on the first five data points leads to an estimate for K_d of 27.1 nM.

Potential for Erroneous Analysis with Conventional Scatchard Analysis

The described loss of ligand activity clearly leads to irreproducible data. The question then arises what influence if any this phenomenon has on the analysis of the data and the results of the data analysis.

In Fig. 4 equilibrium data points are shown in a Scatchard plot for the HEL–D1.3 Fab experiment described above (see Fig. 1A for the experiment layout). Based on all available information about the D1.3 Fab–HEL interaction (2–6) this interaction is a 1:1 interaction. Therefore a linear Scatchard plot is predicted. However, the data points do not appear to exhibit any form of linearity. On the other hand, this cannot really be expected considering that the individual data points are all the results of injections, each of which has experienced a potentially different amount of loss of ligand activity. Typically, nonlinearity of the Scatchard plot is interpreted to indicate a more complex type of interaction than a 1:1 interaction. If it was not known that the D1.3–HEL interaction is a 1:1 interaction we might be misled by the nonlinearity of the Scatchard plot into interpreting the interaction as being more complex than it is.

A second source of potential error could arise if only a small number of data points are acquired or analyzed. In this case the otherwise nonlinear Scatchard plot might in fact appear linear. Depending on the chosen set of points very different equilibrium estimates might result. The data in Fig. 4 are in fact shown divided into four different data subsets of the data set. The experimental design was such that the first set of

data points (Data set 1) corresponded to a set of injections (downward injections) consisting of decreasing analyte concentration values which started at 100 nM and ended at 5 nM. Later in the experiment there was a set (Data set 2) of injections (upward injections) consisting of increasing concentration values starting at 5 nM and ending at 100 nM. This was followed by a repeat set (Data set 3) of downward injections and by another repeat set (Data set 4) of upward injections.

To evaluate the potential for erroneous estimates of equilibrium constants we analyzed various subsets of the measured equilibrium values to determine the corresponding equilibrium constants. In a practical situation these subsets might have been acquired instead of the full set that is presented here. Therefore the analysis of the various subsets can give an indication of the variability of the estimates of the equilibrium constant that might be obtained. Each of these four data sets was analyzed using a standard Scatchard analysis for a 1:1 interaction (Fig. 4). It is clearly seen that the estimated equilibrium dissociation values vary significantly depending on the particular data subset that is used for the analysis. Moreover, the data fits are not satisfactory, especially for Data set 1, which was acquired very early in the experiment while there was significant loss of ligand activity. In particular, the data do not exhibit the linearity that is expected for a 1:1 interaction. That the data exhibit large variability is also indicated by the Scatchard regression lines showing large differences. The estimated equilibrium dissociation constants for the data subsets are given by 8.6 (Data set 1), 2.2 (Data set 2), 2.9 (Data set 3), and 2.8 nM (Data set 4) and show a high variability. Had only a number of data points of Data set 1 been analyzed an even a larger difference in estimated dissociation constants would be apparent. In Fig. 4B it is demonstrated that an analysis of the first 5 data points of Data set 1 would have resulted in an estimated K_d value of 27.1 nM.

Modified Scatchard Analysis and Modeling of Loss of Activity

Under Theory a modification of the standard Scatchard analysis was proposed that takes into account the loss of ligand activity. In Figs. 5 and 7 data fits are shown for the results of an optimization task as described under Theory. To model the loss of sensitivity for the HEL–D1.3 Fab data the decay model

$$R_{max}(t) = \beta e^{-\alpha t} + \gamma,\ t \geq 0, \qquad \text{[Model } D1\text{]}$$

was used and for the I-A^u:10.2.16 Fab data the decay model

$$R_{max}(t) = \beta_1 e^{-\alpha_1 t} + \beta_2 e^{-\alpha_2 t},\ t \geq 0, \quad \text{[Model } D2\text{]}$$

was found to produce adequate fits.

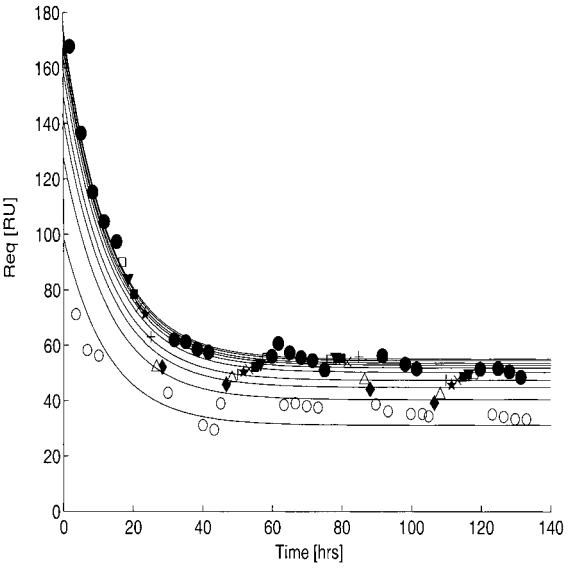

FIG. 5. The result of a curve fit of the model that incorporates exponential loss of activity of the ligand (see Theory) is shown for the complete HEL–D1.3 Fab data set of the experiment described under HEL–D1.3 Fab interaction (Figs. 1A and 2) for flow cell 3. Data points with symbols ○, ◆, △, +, ⋆, ×, ■, ▼, □, and ● correspond to equilibrium values with injection levels 5, 10, 15, 20, 30, 40, 50, 60, 80, and 100 nM, respectively. The solid lines represent the fit of the decay model for the various concentrations to the experimental data. The parameter estimates were obtained by fitting the single exponential decay model $D1$ for R_{max} as presented in Eq. [5] to the data points. The estimated equilibrium dissociation constant is K_d = 4.2 nM. The parameters for the decay model $D1$ are given by α = 0.077 1/h, β = 124.6 RU, γ = 57.2 RU.

For the HEL–D1.3 Fab interaction the equilibrium dissociation constant K_d was determined to be 3.8 nM for the case in which no acid stripping was performed (Fig. 6). The parameters for the decay model were estimated to be β = 315.5 RU, α = 0.034 1/h, and γ = 79.4 RU. For the case in which acid stripping was performed (Fig. 5) an equilibrium dissociation constant of K_d = 4.2 nM was determined and α = 0.077 1/h, β = 124.6 RU, and γ = 57.2 RU. Note that these estimates are in good agreement with the published results for D1.3 Fv fragments and complete antibodies (2–6). The equilibrium dissociation constant for the HEL–D1.3 Fv fragment was determined to be 3 nM using fluorescence spectroscopy (2) and 3.7 nM by calorimetry (5). For a complete D1.3 antibody the equilibrium dissociation constant was determined to be 3.7 nM (3), also using fluorescence spectroscopy.

For the I-A^u:10.2.16 Fab interaction the estimated dissociation equilibrium constant K_d was 170 nM. The parameters for the decay model were estimated to be α_1 = 0.074 1/h, α_2 = 0.0026 1/h, β_1 = 144.8 RU, and β_2 = 271.1 RU.

It is obviously not desirable for routine analyses to have to acquire the large data sets that were discussed

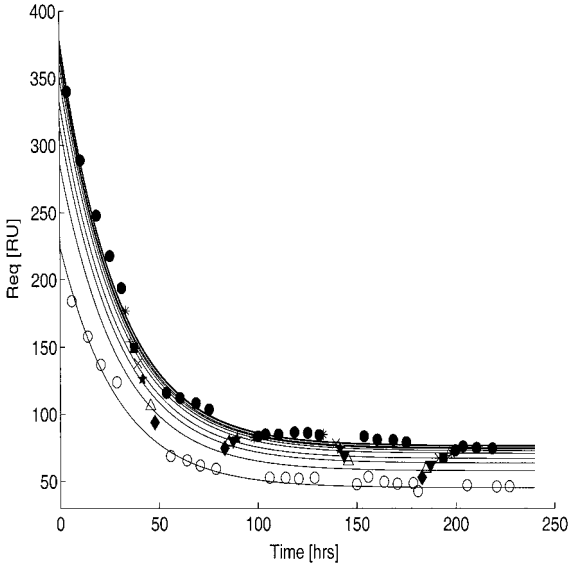

FIG. 6. Data for an experiment for the HEL–D1.3 Fab interaction identical to that in Fig. 5 are presented. The fundamental difference is that during this experiment no stripping of the chip was performed. Data points with symbols ○, ◆, △, ▼, ⋆, ×, ■, +, ∗, and ● correspond to equilibrium values with injection levels 5, 10, 15, 20, 30, 40, 50, 60, 80, and 100 nM, respectively. The solid lines represent the fit of the decay model for the various concentrations to the experimental data. The parameter estimates were obtained by fitting the single exponential decay model $D1$ for R_{max} as presented in Eq. [5] to the data points. The estimated equilibrium dissociation constant is K_d = 3.8 nM. The parameters for the decay model $D1$ were estimated to be β = 315.5 RU, α = 0.034 1/h, γ = 79.4 RU.

above. To deal with this issue an experimental design analogous to that used to acquire the I-A^u:10.2.16 Fab data is suggested.

We would like to obtain a notion of the variability in the estimates that might be introduced by using smaller numbers of repeats of equilibrium estimates to compute the equilibrium constant. In Fig. 8 the results of the processing of subsets of the complete data set are shown. The full experiment consists essentially of a repeat application of block A and block B of analyte injections. In block A a number of repeat injections of the largest and the lowest concentration are carried out (1000, 33, 1000, 33 nM). In block B experiments with all other concentrations are carried out (500, 67, 1000, 133, 33, 250 nM). The complete data set is obtained by carrying out repeats ABABA · · · .

In Fig. 8A the results of data fits are shown for incrementally increasing data sets ABA, ABABA, ABABABA, ... Note that the first three data points were obtained by fitting a single-exponential decay model ($D1$), whereas the remaining data points were obtained by fitting the two-exponential model ($D2$). This switch of model was carried out as for short data sets model $D2$ has too many parameters to lead to a reliable fit. On the other hand, for a larger data set model $D2$ leads to a more satisfactory data fit.

In Fig. 8B the results of data fits are shown for data resulting from the various ABA subsets of the complete data set. The first data point results from the first ABA element in the complete data. The next data point is the K_d estimate for the second ABA data subset of the complete data set, etc.

The results of this analysis show that even with smaller data sets results can be obtained that are consistent with those obtained from a larger data set. However, it is expected that smaller data sets will lead to a larger variance in the estimated parameters. Due to the compensation for the loss of ligand activity a large difference in the estimated equilibrium constants between different data subsets is not seen.

DISCUSSION

The determination of equilibrium binding constants is of central interest in protein–protein interaction studies. The use of the BIAcore instrument provides a convenient way to carry out such studies since labeling of the proteins is not necessary. In this study we have shown that during a BIAcore experiment the activity of the ligand on the chip can decrease significantly during the course of the experiment. In particular, a repetition of the experiment on the same chip can lead to very different results and estimated equilibrium constants. In one of the example systems (HEL–D1.3 Fab interaction) studied here the differences can be up to an order of magnitude. For a 1:1 interaction such as the HEL–D1.3 Fab interaction the Scatchard plot should show a linear relationship between coordinate values.

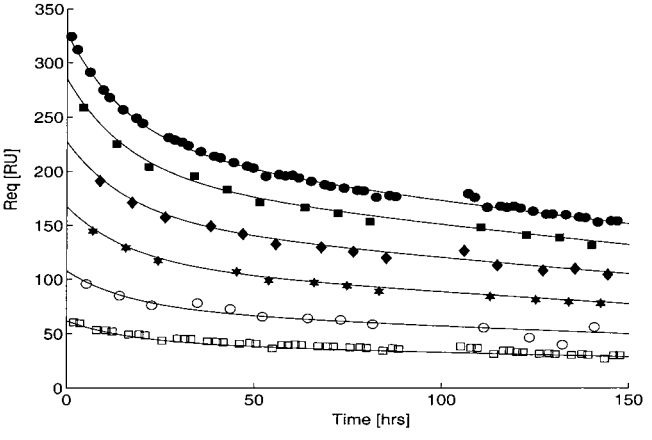

FIG. 7. Data for the I-A^u:10.2.16 Fab interaction (see text) with curve fit for the decay model $D2$ under Theory. Data points with symbols □, ○, ⋆, ◆, ■, and ● correspond to equilibrium values with injection levels 33, 67, 133, 250, 500, and 1000 nM. The estimated decay parameters are given by α_1 = 0.061 1/h, α_2 = 0.0026 1/h, β_1 = 119.7 RU, and β_2 = 263.1 RU. The estimated equilibrium dissociation constant is given by K_d = 170.9 nM.

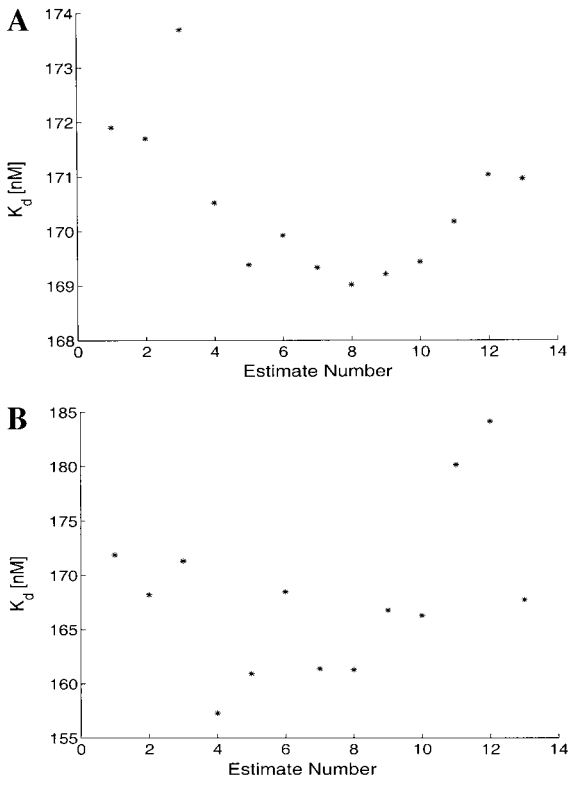

FIG. 8. Results of analysis of data subsets of the data presented in Fig. 7. The full experiment for the study of the I-A^u:10.2.16 Fab interaction consists of a repeat application of blocks A and B of analyte injections. In block A a number of repeat injections of the largest and the lowest concentration (33 and 1000 nM) are carried out. In block B experiments with all concentration levels are carried out. The complete data set is obtained by carrying out repeats ABABA $\cdots$. In (A) the results of data fits are shown for incrementally increasing data sets ABA, ABABA, ABABABA, ... The first three data points were obtained by fitting a single exponential decay model ($D1$), whereas the remaining data points were obtained by fitting the biexponential model ($D2$). In (B) the results of data fits are shown for data resulting from the various ABA subsets of the full data set. The first data point results from the first ABA element in the full data. The next data point is the K_d estimate for the second ABA data subset of the full data set, etc. The mean of the estimated equilibrium constants is K_d = 168.1 nM with standard deviation 7.6 nM.

Clearly in many of the analyses carried out here the expected linearity is not seen. As demonstrated the observed nonlinearity of the graph can lead to a large variability in the estimated equilibrium constant. This is particularly problematic since the observed loss of activity appears to be most severe during the first injections that are performed. It is, however, typically the results from these first injections that are being used for the analysis of the interaction. Of equal importance is that the observed nonlinearity might lead to the erroneous conclusion that a more complex interaction is present than is actually the case.

We have shown that in the example systems discussed here the decay of activity of the ligand is exponential or biexponential in the sense that measured equilibrium values decrease exponentially or biexponentially for repeated experiments as a function of time. The reason for this decrease in activity is not really clear. A likely explanation is that immobilized proteins denature at a steady rate if kept at room temperature as is the case with the protein coupled to the chip. Denaturation of the ligand could also occur as a result of the stripping procedure that is often employed. In order to exclude that stripping is the cause of the phenomenon the HEL–D1.3 Fab experiments presented here have also been carried out without stripping the chip. In fact for the I-A^u:10.2.16 Fab interaction stripping is not possible as the stripping agents immediately denature the I-A^u fragments. A further possible reason for denaturation might have been that amine coupling leads to destabilization of the coupled protein. However, we found that experiments carried out with biotin-coupled ligand show the same exponential decay in the measured equilibrium values of repeated experiments. Similar effects were also observed when the ligand was bound to a C1 sensor chip without dextran.

Nonspecific binding is an effect that can adversely affect the accuracy of the estimate since the measured equilibrium values can also have contributions that are due to this rather than due to the interaction that is of interest. One effect of irreversible nonspecific binding would be that the dissociation curve does not return to the zero level. If there is good reason to believe that nonspecific binding accounts for this nonzero offset it should be subtracted from the measured equilibrium value to correct for nonspecific binding.

In the experiments that were analyzed the decay of the measured equilibrium values could be modeled by an exponential or biexponential decay as proposed under Theory. We have suggested a method that simultaneously estimates the parameters that describe the decay of activity and the desired equilibrium constant. To perform this estimation task we employed a standard gradient-based search algorithm. Using this approach we obtain equilibrium constants that are in excellent agreement with the affinity constants of the HEL–D1.3 antibody (or Fv) interaction determined using solution fluorescence spectroscopy methods (2, 3) and calorimetry (5).

However, care has to be taken with our proposed data analysis approach. For example, if there is no decay in the activity but the data are modeled with the exponential decay ($D1$) as under Theory, the data are in fact overparameterized. This could lead to convergence problems in the optimization routine. Therefore before an analysis is carried out the data should be examined to establish whether there is an appreciable decay of activity. When no appreciable decay is present no decay should be modeled.

An experimental layout was proposed that includes repeats of injections with the same concentration values. This layout has two purposes. First, it allows one to determine whether a problem really exists, i.e., whether loss of ligand activity is a problem during the execution of the experiment. Second, if loss of ligand activity occurs, the acquired equilibrium values can be used in our proposed analysis method to obtain an equilibrium constant with a method that takes into account the loss of ligand activity. In effect what the approach does is simultaneously estimate the rate at which loss of activity occurs and the equilibrium constant. Since more parameters are to be estimated than in a standard Scatchard analysis it is to be expected that in a typical situation more data points, i.e., equilibrium binding values, need to be determined to guarantee that noise does not adversely affect the parameter estimates. For this reason we have proposed an experimental setup in which the complete experiment consists of "blocks" of injections. After each block has been performed the acquired data can be analyzed and it can be determined whether additional blocks of injections should be performed to achieve the required quality of parameter estimates. The analysis of the I-A^u: 10.2.16 Fab interaction shows that it is not necessary to have excessively long data sets to obtain good estimates. While an increase in the number of data points, i.e., the number of measured equilibrium values, will lead to a decrease in the variance of the estimate the observed variance of the estimate based on the shorter data sets would typically be sufficient in many practical situations.

Methods other than the Scatchard-type analysis have been used to determine equilibrium constants. Other methods that are based on the estimation of the equilibrium values, such as isotherm-type plots, appear to have the same problems that are mentioned here in the context of Scatchard analyses. Our data analysis approach is not directly relevant to methods that use measurements of the kinetic association and dissociation constants for the determination of the equilibrium constant. Those methods are, however, often not used due to the inherent problems associated with the accurate determination of kinetic constants (see, e.g., (14)).

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