Analysis of Protein Interactions with Picomolar Binding Affinity by Fluorescence-Detected Sedimentation Velocity

Huaying Zhao¹, Mark L. Mayer², Peter Schuck¹*

¹Dynamics of Macromolecular Assembly Section, Laboratory of Cellular Imaging and Macromolecular Biophysics, National Institute of Biomedical Imaging and Bioengineering, National Institutes of Health, Bethesda, Maryland

²Laboratory of Cellular and Molecular Neurophysiology, Porter Neuroscience Research Center, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland

Supporting Information

Table of Contents:

Method S1: Simulation of kinetically limited binding.

Theory S1: Lower bound for apparent K_D estimates from kinetically limited binding

Figure S1: Raman scattering at the air/water meniscus interface.

Figure S2: Fluorescence signal contribution of unlabeled BSA.

Figure S3: Simulations of kinetically limited binding and impostor equilibrium analyses with short incubation.

Figure S4: Simulations of kinetically limited binding and impostor equilibrium analyses with long incubation.

Figure S5: Simulated binding data after dilution of pre-incubated equimolar stock with short incubation.

Figure S6: Simulated binding data after dilution of pre-incubated equimolar stock with long incubation.

Figure S7: Surface plasmon resonance biosensor experiment of EGFP binding to immobilized mAb.

Supplementary References
**Method S1: Simulation of kinetically limited binding.**

An antibody molecule with two identical binding sites was assumed to have microscopic equilibrium constants $K_D$ of 10 pM, and chemical on-rate constants $k_{on}$ of $10^6$ M$^{-1}$sec$^{-1}$ or $10^7$ M$^{-1}$sec$^{-1}$, and corresponding off-rate constants $k_{off}$ of $10^{-5}$ sec$^{-1}$ or $10^{-4}$ sec$^{-1}$, respectively. The kinetic binding progress between free ligand $L$, free antibody $A$, and single-occupied complex $C$ and double occupied complex $D$ was calculated according to the rate equation

\[
\frac{dL}{dt} = -2k_{on}LA + k_{off}C_1 - k_{on}C_1L + 2k_{off}C_2 \\
\frac{dA}{dt} = -2k_{on}LA + k_{off}C_1 \\
\frac{dC_1}{dt} = 2k_{on}LA - k_{off}C_1 - k_{on}C_1L + 2k_{off}C_2 \\
\frac{dC_2}{dt} = k_{on}C_1L - 2k_{off}C_2
\]

with boundary condition $L(t = 0) = L_{tot}$, $A(t = 0) = A_{tot}$, $C_1(t = 0) = 0$, and $C_2(t = 0) = 0$. Titration experiments were simulated for a ligand at concentration $L_{tot}$ being incubated for time $t_{inc}$ with different concentrations of antibody $A_{tot}$, followed by the determination of a binding signal at time $t_{inc}$ of the form

$$s_w = \frac{s_1[L] + s_{11}[C_1] + 2s_{12}[C_2]}{[L] + [C_1] + [C_2]}$$

, which applies similarly to many biophysical techniques, here mimicking sedimentation velocity of EGFP-IgG interaction with $s$-values $s_1 = 2.6$ S, $s_{11} = 7$ S, and $s_{12} = 8$ S. The initial concentration of ligand $L_{tot}$ was chosen such that equimolar concentrations of ligand and antibody would lead to substantial binding after $t_{inc}$, but still far from saturation. Computations were carried out in MATLAB (Mathworks, Natick, MA).
Theory S1: Lower bound for apparent $K_D$ estimates from kinetically limited binding

We restrict the analysis to 1:1 systems of molecules A and B, at free concentrations $a$ and $b$, respectively, forming a single complex at concentration $c$. Initially, there is no complex, and $a = a_0$ and $b = b_0$. We then allow binding to proceed for only $t_{inc}$ seconds. We are interested in the value of an apparent dissociation constant $K_{D,app} = a(t_{inc})b(t_{inc})/c(t_{inc})$ if evaluated from non-equilibrium concentrations of species at that time, under conditions where less than half of A and B are depleted (and less than half maximum C has formed).

The binding experiment will follow the kinetics

$$\frac{dc}{dt} = k_{on}ab - k_{off}c$$,
$$\frac{da}{dt} = \frac{db}{dt} = -\frac{dc}{dt}$$

with $a = a_0 - c$ and $b = b_0 - c$. We make the approximation $k_{off} = 0$, and, initially far from saturation, consider the reaction to proceed with the initial rate. The resulting concentrations $c'$ follow

$$\frac{dc'}{dt} = k_{on}a_0b_0$$, or $c'(t) = a_0b_0k_{on}t$

It is readily verified that these estimates of species concentrations at all times obey the following relationships relative to the true species concentrations:

$$c' > c, \quad a' = a_0 - c' < a, \quad b' = b_0 - c' < b$$

Furthermore, we want to consider conditions where binding has not yet proceeded to half saturation:

$$a_0/2 < a' = a_0 - c' < a$$
$$b_0/2 < b' = b_0 - c' < b$$

Under these conditions, the ratio of species satisfied the inequality

$$\frac{c}{ab} < \frac{c'}{a_0b_0}$$

from which directly follows a lower limit for the apparent dissociation constant

$$K_{D,app} > \frac{1}{4k_{on}t_{inc}}$$

Notably, this is independent of the true equilibrium dissociation constant.
In the computer simulations Figure S3 and Figure S4 for the bivalent system conducted over a large concentration range, we find $K_{D,\text{app}}$ to satisfy the expected relationship, being 2-4fold above the lower limit.
**Figure S1:** Raman scattering at the air/water meniscus interface. Shown are superpositions of fluorescence radial scans (dots, with increasing color temperature indicating later scans), at 70% PMT voltage and focal depth 3.9 mm, for (A) H$_2$O; (B) H$_2$O with 20% ethanol; or (C) D$_2$O. With volumes of 400 µl inserted in standard Epon double sector centerpieces, the meniscus is located at a radius ~ 6.1 cm, and can be recognized from the transition of baseline signal of ~ 350 counts to the signal inside the solution column of ~ 400 counts for H$_2$O.
Figure S2: Fluorescence signal contribution of unlabeled BSA. (A) Sedimentation coefficient distribution $c(s)$ of 0.1 mg/ml BSA in PBS, recorded with a PMT voltage of 73% at a focal depth of 4 mm. (B) Sedimentation coefficient distributions of 0.5 mg/ml BSA in PBS in the presence of 4 pM – 200 pM EGFP, as indicated in the legend, recorded with a PMT voltage of 75% at a focal depth of 4 mm. $c(s)$ data were normalized to equal total area.
**Figure S3: Simulations of kinetically limited binding and impostor equilibrium analyses with short incubation.** Simulated binding data after incubation for 100 sec (solid blue circles) or 200 sec (open blue circles) in comparison with true equilibrium binding data (open black circles). **Panel A:** Binding isotherms are shown for $k_{on} = 10^6$ M$^{-1}$sec$^{-1}$, at a total ligand concentration of 1 nM (vertical dashed blue line). The best-fit (impostor) equilibrium isotherm to the kinetically limited data with 100 sec incubation is shown as solid red line, leading to an estimate of $K_{D,app} = 6.9$ nM, and the corresponding fit to the 200 sec incubation data is shown as thin dotted red line, leading to an estimated $K_{D,app} = 3.17$ nM. These results are not very sensitive to the value of total ligand concentration: At a 3fold higher $L_{tot}$, the $K_{D,app}$-values are 5.93 and 2.27 nM, and at 3fold lower $L_{tot}$, the $K_{D,app}$-values are 9.2 and 3.8 nM (data not shown). **Panel B:** Binding isotherms are shown for a 10-fold faster reaction with $k_{on} = 10^7$ M$^{-1}$sec$^{-1}$, at a total ligand concentration of 0.1 nM (vertical dashed blue line). Here, the impostor equilibrium fit leads to $K_{D,app} = 0.68$ nM for 100 sec incubation, and 0.32 nM for 200 sec incubation, respectively. Again, these results are not very sensitive to the value of total ligand concentration: At a 3fold higher $L_{tot}$, the $K_{D,app}$-values are 0.60 and 0.25 nM, and at 3fold lower $L_{tot}$, the $K_{D,app}$-values are 0.92 and 0.39 nM (data not shown).
Figure S4: Simulations of kinetically limited binding and impostor equilibrium analyses with long incubation. Analogous to Figure S3, binding data after incubation of 10,000 sec (solid blue circles) or 20,000 sec (open blue circles) in comparison with true equilibrium binding data (open black circles), at a total ligand concentration of 10 pM. Panel A: Binding isotherms are shown for $k_{on} = 10^6 \text{ M}^{-1}\text{sec}^{-1}$. The best-fit (impostor) equilibrium isotherm to the kinetically limited data with 10,000 sec incubation is shown as solid red line, leading to an estimate of $K_{D,app} = 74.4$ pM, and the corresponding fit to the 20,000 sec incubation data is shown as thin dotted red line, leading to an estimated $K_{D,app} = 36.9$ pM. Panel B: Binding isotherms are shown for $k_{on} = 10^7 \text{ M}^{-1}\text{sec}^{-1}$. Here, the impostor equilibrium fit leads to $K_{D,app} = 11.4$ pM for 10,000 sec incubation, and 10.1 pM for 20,000 sec incubation, respectively.
**Figure S5:** Simulated binding data after dilution of pre-incubated equimolar stock with short incubation. For reference, repeated are the titration data from Figure S3 for constant 1 nM or 0.1 nM ligand, respectively, titrated with various concentrations of mAb (solid blue circles, and red line the best-fit impostor equilibrium isotherm). New, shown in magenta, are binding signals from experiments where a 100 pM equimolar mixture is pre-incubated for 1 hour (magenta stars) or 2 hours (green triangles), followed by dilution to various equimolar final concentrations and relaxation for 100 sec before measurement. Similarly, in green are binding signals at a lower, 10 pM equimolar mixture, pre-incubated for 1 hour (green stars) or 2 hours (green triangles), followed by dilution to various equimolar final concentrations and relaxed for 100 sec. **Panel A:** $k_{on} = 10^6 \text{M}^{-1}\text{sec}^{-1}$; **Panel B:** $k_{on} = 10^7 \text{M}^{-1}\text{sec}^{-1}$.

None of the experiments with the pre-equilibrated mixture has sufficient time to relax to the final dilution during the experiment time. The discrepancy between the dilution and titration isotherm signifies the impostor non-equilibrium analysis.
Figure S6: Simulated binding data after dilution of pre-incubated equimolar stock with long incubation. For reference, repeated are the titration data from Figure S4 for constant 10 pM ligand titrated with various concentrations of mAb (solid blue circles, and red line the best-fit impostor equilibrium isotherm). New, shown in magenta, are binding signals from experiments where a 100 pM equimolar mixture is pre-incubated for 1 hour (magenta stars) or 2 hours (magenta triangles), followed by dilution to various equimolar final concentrations and relaxation for 10,000 sec before measurement. Similarly, in green are binding signals at a lower, 10 pM equimolar mixture, pre-incubated for 1 hour (green stars) or 2 hours (green triangles), followed by dilution to various equimolar final concentrations and relaxed for 10,000 sec. Panel A: $k_{on} = 10^6$ M$^{-1}$sec$^{-1}$; Panel B: $k_{on} = 10^7$ M$^{-1}$sec$^{-1}$.

In Panel A, the pre-equilibrated mixture at 100 pM has attained equilibrium (magenta), but cannot relax in the given time to the new equilibrium after dilution. By contrast, the 10 pM mixture has not yet reached equilibrium (difference between green triangles and stars for 1 or 2 hours pre-equilibration), and its dilution to different experimental concentrations is far from equilibrium. In Panel B, the pre-equilibrated stock mixture has attained equilibrium in both cases (both arriving at similar saturation of the complex), but the dilution time is still not sufficient to allow the final binding data to reflect true equilibrium. However, here similar fraction bound are obtained at final 10 pM equimolar concentration for the direct titration and both pre-equilibration/dilution experiments. This is an indication that the binding experiment is close to equilibrium, with binding constants $K_{D,app}$ nearly identical to the true $K_D$. Under the given experimental conditions, at $k_{on} = 10^8$ M$^{-1}$sec$^{-1}$, equilibrium is practically achieved in all titration and dilution configurations (data not shown).
Figure S7: Surface plasmon resonance biosensor experiment of EGFP binding to immobilized mAb.

SPR biosensor binding experiments were performed in a Biacore 3000 (GE Healthcare, Piscataway, NJ) with a sensor chip CM5. Anti-GFP mAb (50 µg/ml at pH 5.5 in acetate buffer) was immobilized on the sensor surface using standard amine coupling procedures to a level of 570 RU. Phosphate saline buffer containing 0.005% P20 was the running buffer, at a flow rate of 5 µl/min. A concentration series of EGFP (0.03, 0.10, 0.30, 1.0, 3.0, 10, 30 and 100 nM) prepared in the running buffer were injected in each cycle of the method. Binding was observed for 20 min, followed by a long dissociation phase of 2 h in order to maximize the measured dissociation. Reversible surface regeneration was achieved with a 1 min injection of 10 mM Glycine-HCl at pH 1.8. Data analysis was performed in EVILFIT with both a discrete single site model and surface site distribution model. Panel A: SPR binding data at different concentrations (blue to green) and best-fit binding kinetics predicted by pseudo-first-order kinetics (red lines) as expected for an immobilized monoclonal IgG binding soluble antigen. Panel B: Same data as in (A), and best-fit binding kinetics predicted by a model for a continuous distribution of sites with different affinity and rate constants shown in (C) (red lines). Panel C: Calculated distribution of binding sites with different affinity and kinetic rate constants. The color contour is scaled such as to show higher population of sites by higher color temperature, as indicated by the color bar. The circles are grid points in the calculation of the distribution, the grey vertical lines indicate experimental concentrations used, and the horizontal grey lines indicate the highest and lowest koff that is well-determined by the data (based on the delay from the onset of dissociation to the first data point in the dissociation phase, and the total observation time in the dissociation phase).
Supplementary References:

(1) Schuck, P.; Boyd, L. F.; Andersen, P. S. Curr. Protoc. Cell Biol. 1999, 17, 17.6.1–17.6.22.

(2) Svitel, J.; Balbo, A.; Mariuzza, R. A.; Gonzales, N. R.; Schuck, P. Biophys. J. 2003, 84, 4062–4077.