Chapter 3

Radioligand Binding Assays for Determining Dissociation Constants of Phytohormone Receptors

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Abstract

In receptor–ligand interactions, dissociation constants provide a key parameter for characterizing binding. Here, we describe filter-based radioligand binding assays at equilibrium, either varying ligand concentrations up to receptor saturation or outcompeting ligand from its receptor with increasing concentrations of ligand analogue. Using the auxin coreceptor system, we illustrate how to use a saturation binding assay to determine the apparent dissociation constant (K_D') for the formation of a ternary TIR1–auxin–AUX/IAA complex. Also, we show how to determine the inhibitory constant (K_i) for auxin binding by the coreceptor complex via a competition binding assay. These assays can be applied broadly to characterize a one-site binding reaction of a hormone to its receptor.

Key words Radioligand binding, Hormone receptor, Binding affinities, Saturation binding, Competition binding

1 Introduction

Radioligand binding assays have been widely used in biochemistry and pharmacology to determine the binding affinities of ligands, such as small molecules, nucleic acids, and peptides to their receptors. Intermolecular forces, such as ionic bonds, hydrogen bonds, and van der Waals forces, define affinity binding. Affinity, as a measure of how "tightly" a ligand binds to its receptor, is described using the equilibrium dissociation constant K_D. Based on the law of mass action, K_D of a reversible binding reaction, Receptor (R) + Ligand (L) $\rightleftarrows$ Complex (RL) is defined as:

$$K_D = \frac{[R][L]}{[RL]}$$

at equilibrium at a given temperature. This means, if complex concentration at equilibrium is high and only little free ligand and free receptor is left, the K_D is a low value signifying high affinity

binding. In turn, if free ligand and receptor outweigh the complex at equilibrium, i.e., binding affinity is low, K_D will take on a high value.

For a typical saturation binding assay (Fig. 1a), the total receptor concentration $[R_{total}]$ is kept low and constant, while different concentrations of ligand $[L_{total}]$ are tested. The amount of total receptor used is a sum of free receptor concentration and complex concentration at equilibrium: $[R_{total}] = [R] + [RL]$. Also, $[R_{total}]$ is
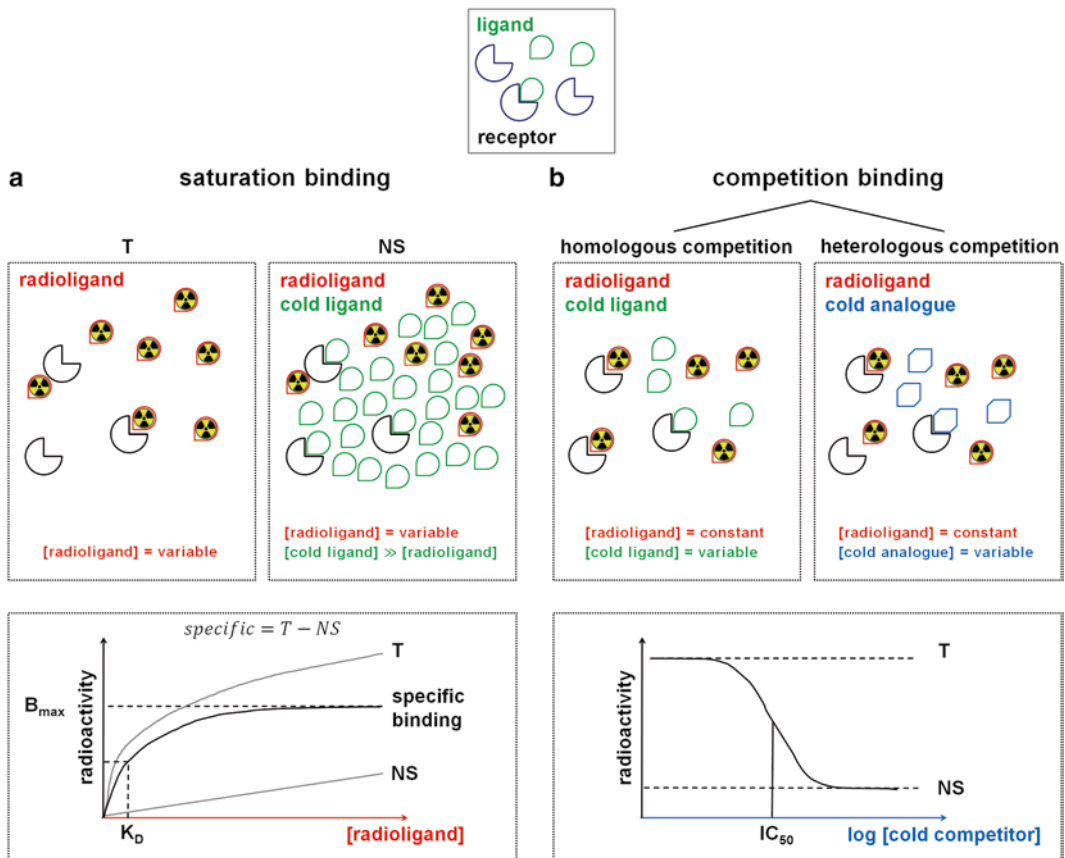
Fig. 1 Binding of ligand to its receptor can be assessed via two types of radioligand binding assays: Saturation and Competition Binding. (**a**) Saturation binding assays allow measuring of total (T) and nonspecific (NS) binding. To measure T and NS binding, receptor concentration is kept constant, while radioligand concentration is varied. NS samples are prepared with excess of unlabeled (cold) ligand. Specific binding results from subtracting NS from T, and will be plotted against radioligand concentration. Nonlinear regression allows determination of the dissociation constant K_D. (**b**) Competition binding assays can be performed as homologous (unlabeled competitor is identical with radioligand) or heterologous (unlabeled competitor is an analogue of the radioligand) assays. In both cases, receptor and radioligand concentrations are kept constant, while the concentration of unlabeled (cold) competitor is varied. Plotting the measured radioligand bound against logmolar concentration of cold competitor allows nonlinear regression to determine half-maximal inhibitory concentration IC_{50} of competitor

equivalent to maximum binding B_{max}, which will be determined in the experiment. Consequently, the equation for K_{D} can be rearranged to:

$$K_{\text{D}} = \frac{\left(B_{\text{max}} - [RL]\right) \cdot [L]}{[RL]} = \frac{B_{\text{max}} \cdot [L]}{[RL]} - [L]$$

$$[RL] = \frac{B_{\text{max}} \cdot [L]}{K_{\text{D}} + [L]}$$

Assuming only a small fraction of ligand is binding to the available receptor, so that $[L_{\text{total}}] \approx [L]$, the resulting equation is:

$$[RL] = \frac{B_{\text{max}} \cdot [L_{\text{total}}]}{K_{\text{D}} + [L_{\text{total}}]}$$

where $[L_{\text{total}}]$ is a known variable and B_{max} and K_{D} are specific constants we aim to determine. To quantify $[RL]$ as a function of the varied $[L_{\text{total}}]$, a radio isotope-labeled form of ligand (hereafter referred to as radioligand) is utilized, and the RL complex is immobilized on a glass fiber filter. Unbound radioligand is rapidly washed out with cold buffer to minimize disturbance of equilibrium. This is best accomplished using a suitable vacuum manifold or harvester. Take into account that if a given receptor–ligand complex exhibits a high dissociation rate, washing procedures can easily disturb the equilibrium, and might result in failure of capturing the true amount of the RL complex. In this case, consider alternative methods [1], e.g., surface plasmon resonance [2], isothermal titration calorimetry [3], fluorescence polarization [4], or thermophoresis [5]. With the assumption that radioligand can bind nonspecifically, for instance to nonreceptor sites on the protein, the filter paper, the tubes, etc., reactions need to be prepared in two sets: (1) containing only receptor and radioligand to obtain total (T) binding and (2) containing receptor and radioligand with the addition of excess of unlabeled ligand to obtain nonspecific (NS) binding. Subtraction of NS from T binding will result in specific binding values that are plotted against ligand concentration. Nonlinear regression with the equation derived above will yield a specific K_{D} (binding affinity) of a ligand for the receptor or vice versa.

Often, ligand analogues, i.e., compounds that bind to the same binding site in the receptor, are not available in a radiolabeled form. In this case, heterologous competition binding experiments can be carried out and allow for determination of inhibitory constant K_{i}, i.e., the K_{D} of binding of the analogue to the receptor. Note that beside heterologous competition assays, one can also perform homologous competition assays with the identical, unlabeled form of radioligand.

For a competition binding experiment (Fig. 1b), the total receptor concentration and radioligand concentration [*Radioligand*] are kept constant, while the concentration of the unlabeled competing analogue [*competitor*] is varied from zero to excess. A data curve for outcompetition of radioligand from the receptor binding site is obtained, and usually plotted against logmolar concentration of competitor. The curve follows the equation:

$$[RL] = NS + \frac{T - NS}{1 + 10^{([\text{competitor}] - \log IC_{50})}},$$

where plateaus at both ends of the curve correspond to T and NS. As mentioned above, here too, the difference between T and NS binding gives specific binding. The concentration of competitor that is needed to reduce specific binding by half is referred to as half-maximal (or 50%) inhibitory concentration IC_{50}.

The inhibitory constant K_i can be determined from the IC_{50} via the Cheng–Prusoff equation [6]. This requires at least an estimation of K_D of radioligand for the receptor.

$$K_i = \frac{IC_{50}}{1 + \dfrac{[\text{Radioligand}]}{K_D}}$$

The methods of saturation and competition binding described here, further assume a one-site binding and no cooperativity. If there is more than one binding site to the receptor, or a more complex molecular mechanism, one can still apply one-site models, but has to refer to the apparent dissociation constant $K_D{}'$ and interpret results appropriately. For more detailed information, we refer the reader to the vast literature resources on binding theory, for instance [7–12].

Various phytohormone receptor systems have been characterized using recombinant receptor and radiolabeled phytohormone or their analogues, including receptors for auxin [13], brassinosteroids [14, 15], jasmonic acid [16], salicylic acid [17, 18], gibberellins [19], and abscisic acid [20].

Our studies on the auxin sensing mechanism have included in vitro radioligand binding assays, which have served as powerful tool to understand how the ligand auxin might be bound by its receptor in vivo [13]. Auxin sensing requires the concerted action of F-box proteins TIR1/AFB1-5 and their targets for degradation, AUX/IAA transcriptional repressors [21]. TIR1/AFBs and AUX/IAAs act together to perceive auxin, and transmit the auxin signal, which in fact triggers changes in gene expression thereby modulating cell division, elongation, and differentiation [21–24]. Auxin occupies a pocket at the interface between TIR1 and AUX/IAAs proteins, so that auxin and AUX/IAA binding sites are spatially

connected. Auxin ultimately acts as molecular glue, increasing TIR1–AUX/IAA affinity by cooperatively binding to both proteins [25]. Since an auxin receptor with full ligand-binding capability is constituted by a TIR1/AFB and an AUX/IAA protein, TIR1/AFB, and AUX/IAA together are referred to as a coreceptor system for auxin sensing [13]. In binding assays for assessing auxin sensing by the coreceptor system, all assumptions: equilibrium reached, no ligand depletion, one-site binding, no cooperativity are met. Yet, it needs to be taken into account that the precise molecular mechanism of binding hierarchy of auxin to the single receptor components is still unclear and, therefore, radioligand binding assays described here indicate an apparent K_D' for formation of a TIR1–auxin–AUX/IAA ternary complex.

2 Materials

2.1 Saturation and Competition Binding Reactions

1. Binding buffer: 50 mM Tris–HCl pH 8.0, 200 mM NaCl, 10% Glycerol, 0.1% Tween-20. Always prepare fresh, filter through a 0.45 μm pore size membrane, and cool at 4 °C. Freshly add 1 mM PMSF and Roche cOmplete EDTA-free protease inhibitor cocktail before use.

2. Receptor: Recombinantly expressed and purified protein. Proteins *a* and *b* are needed for a coreceptor system. In case of auxin sensing:

   (a) TIR1–ASK1 complex at ≥1 mg/mL, usually stored in 50 mM Tris–HCl pH 8.0, 200 mM NaCl, 10% Glycerol.

   (b) AUX/IAA at ≥0.2 mg/mL in binding buffer (*see* **Note 1**).

3. Radioligand: Tritiated indole-3-acetic acid [5-³H] (hereafter ³H-IAA), e.g., with specific activity 25 Ci/mmol at 1 mCi/mL concentration, dissolved in ethanol. For convenient pipetting, the concentration of the radioligand stock you acquire from the provider should be at least tenfold higher than the maximal concentration you will test in the assay. Store at –20 °C in the dark.

4. Unlabeled ligand or analogue (cold competitor): 100 mM indole-3-acetic acid (cold IAA stock) dissolved in absolute ethanol is used for saturation and homologous competition binding experiments. Other auxinic compounds (agonists and antagonists) at 100 mM concentration dissolved in absolute ethanol are used for heterologous competition binding experiments. Alternatively, cold competitor stock solutions can be prepared using DMSO as solvent.

5. 5 mL reaction tubes (75 × 12 mm) or any harvester-suitable tubes ≥1 mL.

6. Ice container holding sample racks for harvester.

7. Orbital shaker.

2.2 Quantifying Bound Radioligand

1. Wash buffer: 50 mM Tris–HCl pH 8.0, 200 mM NaCl. Filter wash buffer through a 0.45 μm pore size membrane and cool at 4 °C. Approximately 10 mL of wash buffer per sample should be calculated. Also include additional volume for priming and rinsing the harvester taking into account the internal volume of the device (*see* **Note 2**).

2. Filter paper buffer: Use wash buffer and add 0.5% (v/v) of a 50% (w/v) aqueous polyethylenimine (PEI) or polyaziridine solution. PEI is a cationic polymer and filters coated in PEI have increased binding.

3. Glass fiber filter paper, e.g., Whatman GF/B paper (fired).

4. Harvester or vacuum manifold for filter-binding assays, e.g., Brandel 24-sample system.

5. Forceps.

6. Scintillation liquid for universal application including aqueous samples or for glass fiber filters (e.g., National Diagnostics EcoScint, Zinsser Analytic Filtersafe) (*see* **Note 3**).

7. Liquid scintillation vials, e.g., Beckman Mini PolyQ Vials.

8. Liquid scintillation counter, e.g., Beckman LS6500.

2.3 Data Analysis

1. GraphPad Prism5 or higher. Alternatively, any comparable data analysis software that includes or can be configured to perform one-site saturation binding (hyperbola) and one-site competition IC_{50} regression.

3 Methods

Perform all procedures on ice or in a cold room.

3.1 Radioactivity Calculations

To calculate the remaining concentration of radioactivity c_{stock} in the ³H-IAA stock solution, determine the decay time t passed since determination of the given specific activity (*see* **Note 4**).

1. Calculate remaining fraction of radioactivity after decay F using the following equation:

$$F = e^{\left(\frac{-\ln 2}{t_{0.5}} \cdot t\right)}$$

As we use ³H-IAA here, $t_{0.5}$ is 4537 days.

2. Calculate the original concentration c_0 from the specific activity A and the radioligand concentration c_R (*see* **Note 4**), using the following equation:

$$c_0 = \frac{c_R}{A}$$

(*see* **Note 5**).

Table 1
Example of a sample set for saturation binding

	Triplicates			[radioligand] (nM)	Components
T	c_1^A	c_1^B	c_1^C	600	Receptor + radioligand
T	c_2^A	c_2^B	c_2^C	300	Receptor + radioligand
T	c_3^A	c_3^B	c_3^C	150	Receptor + radioligand
T	c_4^A	c_4^B	c_4^C	100	Receptor + radioligand
T	c_5^A	c_5^B	c_5^C	60	Receptor + radioligand
T	c_6^A	c_6^B	c_6^C	30	Receptor + radioligand
T	c_7^A	c_7^B	c_7^C	15	Receptor + radioligand
NS	c_1^A	c_1^B	c_1^C	600	Receptor + radioligand + excess unlabeled ligand
NS	c_2^A	c_2^B	c_2^C	300	Receptor + radioligand + excess unlabeled ligand
NS	c_3^A	c_3^B	c_3^C	150	Receptor + radioligand + excess unlabeled ligand
NS	c_4^A	c_4^B	c_4^C	100	Receptor + radioligand + excess unlabeled ligand
NS	c_5^A	c_5^B	c_5^C	60	Receptor + radioligand + excess unlabeled ligand
NS	c_6^A	c_6^B	c_6^C	30	Receptor + radioligand + excess unlabeled ligand
NS	c_7^A	c_7^B	c_7^C	15	Receptor + radioligand + excess unlabeled ligand

3. Calculate remaining concentration c_{stock} by multiplying the original concentration c_0 with remaining fraction of radioactivity after decay F.

$$c_{stock} = F \cdot c_0$$

3.2 Saturation Binding Experiment

1. Label tubes for reactions in triplicates for at least five different concentrations of ^{3}H-IAA. The concentrations should lie around the expected K_D and include high (i.e., up to tenfold K_D) concentrations to approximate B_{max}. Of each reaction you will have to prepare one set of tubes for measuring total binding and another set for measuring nonspecific binding. For example, you want to test seven concentrations of radioligand concentration c_1–c_7 in triplicates (A, B, and C). For every cn^A, cn^B, cn^C you will have to prepare a total binding reaction (T) and a nonspecific binding reaction (NS), resulting in number of samples $N = 42$ samples (*see* Exemplary Sample Table 1).

2. To have 100 µL reactions that include: binding buffer, 10–15 nM TIR1–ASK1 complex and 1–5 µM AUX/IAA, prepare a $N + 3$-times master mix and distribute the appropriate sample volume to individual tubes.

3. Using the 100 mM cold IAA stock, prepare a 25 mM predilution in binding buffer. For NS binding samples, add 4 μL of the 25 mM predilution and gently mix reaction, to obtain 1 mM cold IAA concentration per reaction (>1000× excess unlabeled ligand). Keep in mind though that the excess of unlabeled ligand is relative to the maximal radioligand concentration in your assay. For T binding samples, add equivalent volume of binding buffer.

4. Prepare a dilution series of ³H-IAA stock that allows you to pipet the same amount of volume, e.g., 5 μL, per sample obtaining the desired final concentration of radioligand.

5. After all components have been added to the 100 μL reaction, incubate on ice or in a 4 °C cold chamber with gentle shaking. This incubation allows the binding reaction between ligand and receptor to reach equilibrium. Usually 30–60 min suffice, but incubation time may vary depending on binding kinetics. Note that K_D is temperature-dependent.

3.3 Competition Binding Experiment

1. Label tubes for reactions in triplicates for at least eight different cold competitor concentrations spanning approximately eight (8) orders of magnitude around the 50 % inhibitory concentration IC_{50}. Include a sample without cold competitor for determining T binding, and another one with excess of cold competitor (10,000-fold K_D) for NS binding. T and NS are required for reliable regression. The concentration of radioligand should be $\geq K_D$, and give at least a 1000 cpm signal for T, to ensure a sufficient dynamic range.

2. Prepare 100 μL-reactions mixing binding buffer, 10–15 nM TIR1–ASK1 complex, 1–5 μM AUX/IAA and appropriate concentration of ³H-IAA (see above). We recommend to prepare an $N+3$-times master mix. Distribute the appropriate sample volume to individual tubes.

3. Using the 100 mM cold competitor stock, prepare a 10 mM predilution in binding buffer. From that, prepare a dilution series of cold competitor that allows you to pipet the same amount of volume, e.g., 5 μL, per sample obtaining the desired final concentration of competitor. For T, add the equivalent volume of binding buffer.

4. After all components have been added to the 100 μL reaction, incubate on ice with gentle shaking. This incubation allows equilibrium formation. Usually 30–60 min suffice, but incubation time may vary depending on binding kinetics. Note that K_D is temperature-dependent.

3.4 Quantifying Bound Radioligand

1. Prepare the required number of glass fiber filter papers by soaking them in filter paper buffer at room temperature for 30 min.

2. Rinse the harvester with water and prime with wash buffer.

3. Insert a filter paper and aspirate the samples through the filter. Immediately wash the filter two times with 2 mL and once with 4 mL chilled wash buffer.

4. Carefully remove the filter paper with forceps from the harvester and transfer the filter discs corresponding to your samples to scintillation vials.

5. If due to the number of samples more than one filter paper is required, wash the harvester twice with 4 mL wash buffer, before inserting the next filter.

6. In addition, collect few filter discs corresponding to empty sample tubes to measure background signal.

7. Add 4 mL of scintillation liquid per vial, cap vial and ensure complete immersion of filter disc by shaking or vortexing vigorously.

8. Incubate for several hours or overnight at room temperature.

9. Perform scintillation counting for ^3H isotope, e.g., 1 min counting time.

3.5 Data Analysis

1. After obtaining counts per minute (cpm) values from scintillation counting, subtract background counts from all samples.

3.6 Analysis for Saturation Binding

1. Subtract cpm values for NS from cpm values for T to obtain specific binding values in cpm.

2. Plot mean cpm values for specific binding (y) against radioligand concentration (x).

3. Perform nonlinear regression using the following equation:

$$y = \frac{B_{\max} \cdot x}{K_D + x}$$

4. Resulting hyperbola will approximate $B_{\max}$.

5. K_D can be obtained by solving the resulting regression equation for x at $y = 0.5 B_{\max}$.

3.7 Analysis for Competition Binding

1. Plot mean cpm values (y) against logarithm of cold competitor concentration (x).

2. Perform nonlinear regression using the following equation:

$$y = NS + \frac{T - NS}{1 + 10^{(x - \log IC_{50})}}$$

3. From the regression you obtain $\log IC_{50}$. Calculate the K_i for binding of the cold competitor to the receptor according to Cheng–Prusoff [6]:

$$K_i = \frac{IC_{50}}{1 + \dfrac{[\text{Radioligand}]}{K_D}}$$

Be aware that as mentioned before one needs to have an estimate for a K_D for binding of the radioligand to the receptor.

See **Note 6** for troubleshooting on data fitting.

4   Notes

1. We recommend using fresh, affinity-purified protein samples. Do not freeze, keep on ice, and use latest 5 days after purification.

2. We typically prepare ≥ 1.5 L of wash buffer for one set of 24 samples for a Brandel 24-sample harvester, 3 L are sufficient for up to four sets of 24 samples and so on.

3. The volume of scintillation liquid per sample varies depending on size of the filter discs obtained from the harvester system and the vials used in the liquid scintillation counter. We use 4 mL per filter discs obtained from a Brandel 24-sample harvester system in Beckmann PolyQ Mini Vials to completely immerse the filter disc.

4. Information on specific activity should be provided in a technical data sheet shipped with the radioligand.

5. With c_R given in mCi/mL and A given in mCi/mmol, the resulting c_0 will be in mM.

6. If fitting the data does not result in reliable curves and reasonable K_D values, consider the following:

   (a) Is the radioligand and/or unlabeled ligand or analogue still intact? Auxinic compounds, e.g., are highly photolabile and decompose over extended time even when stored at dark, or at $-20\,°C$.

   (b) Do your receptor preparations contain enough active species? Ideally have an alternative assay at hand to check integrity and/or activity of your protein preparations.

   (c) Do the assumptions made apply to your system? If cooperativity or other special scenarios apply to your binding reaction, consult the appropriate models for fitting the data. If you are not sure if equilibrium has been reached, try to determine the half-life of the complex and extend incubation time accordingly if necessary.

Acknowledgments

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