Protein–ligand interactions

To better understand the interaction between the enzyme and its substrate, it is necessary to have an idea of the interaction between any ligand and its protein receptor. In fact, what characterizes this interaction, more than the kinetics, is the number of ligand binding sites on the receptor protein, which can generally be quantified by measuring the concentration of ligand bound to the protein at thermodynamic equilibrium.

1. Definition :

1. 1. Ligand :

A ligand is a small molecule that is specifically recognized and bound by a protein molecule. Ligand binding occurs non-covalently, through electrostatic forces, Van der Waals forces, hydrogen bonding, and sometimes hydrophobic interactions. Ligand binding occurs at a specific site, with close complementarity between the ligand and the protein surface.

The ligand can be : an ion (metallic or other), a coenzyme, a hormone, a growth factor, a cytokine, a neurotransmitter, a nucleic acid, or an allosteric effector (activator or inhibitor). The receptor protein can be : an enzyme, a membrane receptor, histones, or an allosteric enzyme.

1. 2. Binding site and active site :

The interaction between the protein and its ligand requires the latter to bind to a specific area of the protein called the binding site.

In the case of enzymes, the different sites existing on the surface of the apoenzyme are :

- Substrate recognition site(s).
- Coenzyme binding site.
- Mineral cofactor binding site(s).
- Catalytic transformation zone.
- Regulation site.
- Cell structure binding site.

2. Protein–ligand interaction :

The binding of a ligand (L) to its protein receptor (R) is reversible and can be represented by :

$$R + L \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} RL \quad \text{.....(1)}$$

The **rate** at which the ligand binds to its receptor depends on the concentrations of the ligand (L) and the receptor (R) :

$$v_1 = k_1 [R] [L]$$

The dissociation rate depends on the concentration of the ligand-receptor (LR) complex :

$$\mathbf{v_2 = k_{-1}\,[RL]}$$

When equilibrium is reached, $\mathbf{v_1 = v_2}$ and we can write :

$$k_1\,[R]\,[L] = k_{-1}\,[RL]$$

$$\mathbf{[R]\,[L] / [RL] = k_{-1} / k_1 = K_D}$$

$$\mathbf{[RL] / [R]\,[L] = k_1 / k_{-1} = K_A}$$

Where :

K_D is the dissociation equilibrium constant (mole.l $^{-1}$).
K_A is the association equilibrium constant (l.mole $^{-1}$).
[R] receptor protein concentration.
[L] free ligand concentration.
[RL] the bound ligand concentration.

K_D is smaller, or K_A is larger, when the ligand-protein bond is stronger (RL complex less easily dissociable).
If the protein (P) has (n) binding sites (R) for the ligand (L), the reaction becomes:

$$\mathbf{nR + nL \rightleftharpoons nRL} \quad \text{.....(2)}$$

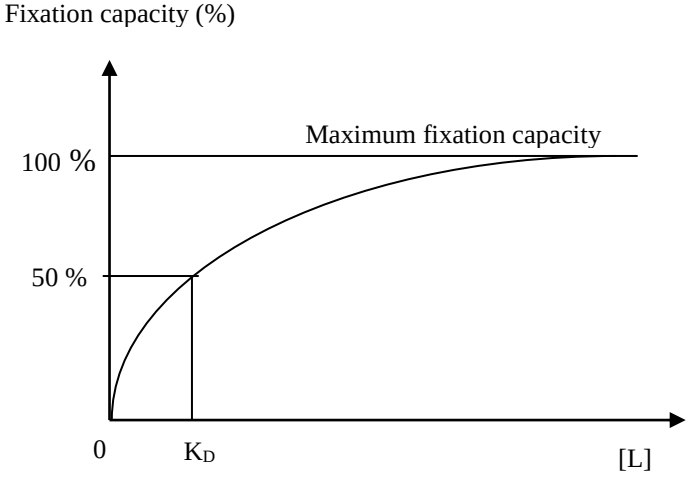
Concept of affinity :

The affinity of a receptor is expressed by the dissociation constant of the ligand-receptor complex (**K_D**), whose value ranges from 10^{-12} to 10^{-4} M, depending on the case. The lower this constant, the higher the affinity of the receptor for its ligand.

3. Graphical representations of protein-ligand interaction :

Consider [Pt] and [Rt] to be the molar concentrations of total protein and all binding sites, respectively :

The concentration of free ligand (K_D) required to saturate half of the receptor sites allows the affinity of the receptor for the ligand to be assessed. The lower this concentration, the higher the affinity of the receptor for the ligand.



3. 1. Proteins with one binding site :

The binding of a ligand to a protein that has a binding site (a receptor, monomeric enzyme) is expressed by :

$$R + L \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} RL \quad \text{so} \quad [R]\,[L] \,/\, [RL] = k_{-1} / k_1 = \mathbf{K_D}$$

$$\text{and} \quad \mathbf{Keq} = [RL] / [R]\,[L] = \mathbf{K_A}$$

Knowing that [R], [L], and [RL] are defined when thermodynamic equilibrium is achieved.

We have : $\mathbf{[R_t] = [R] + [RL]}$ from which $[R] = [R_t] - [RL]$

Therefore : $[R] = [R_t] - [RL]$ ……**(a)**

NB : (a) *is called the* ***protein conservation relation***.

We know that :

$K_A = [RL] / [R]\,[L]$ so $K_D = [R]\,[L] / [RL]$ ……**(b)**

Let's combine (a) and (b) :

$$K_D = ([R_t] - [RL])\,[L] / [RL]$$

$$K_D\,[RL] = [R_t]\,[L] - [RL]\,[L]$$

$$[RL]\,(K_D + [L]) = [R_t]\,[L]$$

$$\mathbf{[RL] = [R_t]\,[L] / (K_D + [L])}$$

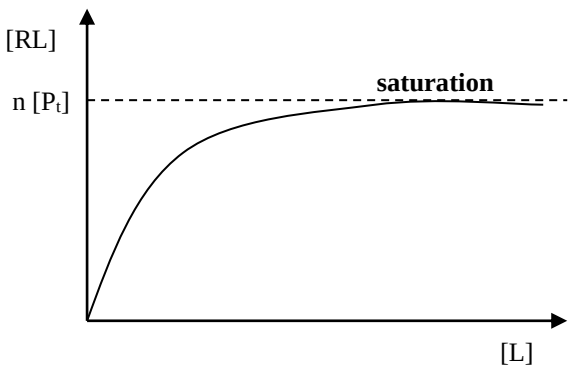
Therefore, [RL] depends on K_D, [L], and $[R_t]$ according to a hyperbolic function. This is called ***Michaelian saturation***.

As : $[R_t] = [P_t]$ so $\mathbf{[RL] = [P_t]\,[L] / (K_D + [L])}$……**(1)**

Thus : **[RL] / [Pt] = [L] / (KD + [L])** is called the ***saturation function*** **(Ў)** as it provides information on the saturation degree of the protein. It varies between 0 and 1.

The Michaela representation

It consists of relating [RL] to [L]. The resulting graph is a hyperbole.



3. 2. Proteins with several binding sites :

In this case the sites can be :

Dependent sites : where ligand binding at one site depends on the saturation state of other sites (as in allosteric enzymes).

Independent sites : where ligand binding to one site is independent of the saturation state of the other sites.

Independent and equivalent sites : each site has the same dissociation constant (K_D).

Independent and non-equivalent sites : the sites have different dissociation constants ($K_{D1} \neq K_{D2} \neq K_{D3} \neq \ldots$).

3. 2. 1. Independent and equivalent sites :

The protein has (n) equivalent binding sites. This is frequently encountered in oligomeric enzymes that have a binding site for the substrate on each monomer. The reaction is represented by :

$$nR + nL \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} nRL$$

So equation (1) becomes :

$$\mathbf{[RL] = n\,[P_t]\,[L] \,/\, (K_D + [L]) = n\,[P_t]\,\bar{y}} \ldots\ldots\ldots(2)$$

$$[RL] \,/\, [P_t] = n\,[L] \,/\, (K_D + [L]) = \mathbf{n\,\bar{y}}$$

where (**n$\bar{y}$**) is the ***average number*** of sites occupied by protein.

Linearization methods (of the Michaelian representation) can be used to determine the parameters that characterize ligand binding to the receptor (n).

In fact, there are two most commonly used representations :

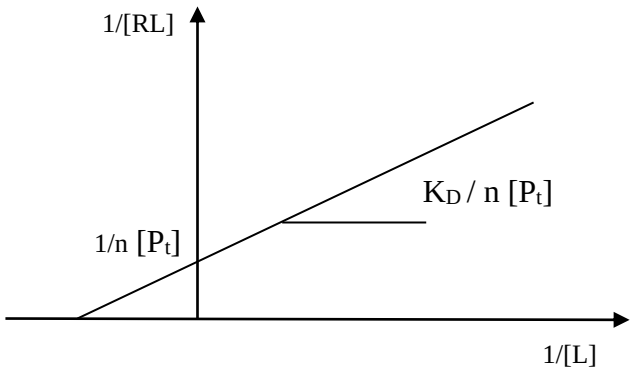
KLÖTZ representation

We have the equation (2) : $[RL] = n\,[P_t]\,[L] \,/\, (K_D + [L])$

$$1 \,/\, [RL] = K_D \,/\, n\,[P_t]\,[L] + 1/\, n\,[P_t]$$

$$\mathbf{1/[RL] = 1/[L]\,(K_D \,/\, n\,[P_t]) + 1/\, n\,[P_t]}$$

Therefore, KLÖTZ's representation consists of relating (1/[RL]) to (1/[L]).



The graph obtained is a straight line with slope ($1/K_A$ n $[P_t]$) or (K_D/n $[P_t]$) and y-intercept at the origin ($1/n$ $[P_t]$).

SCATCHARD Representation

We have :

$$K_D = (n [P_t] – [RL]) [L] / [RL]$$

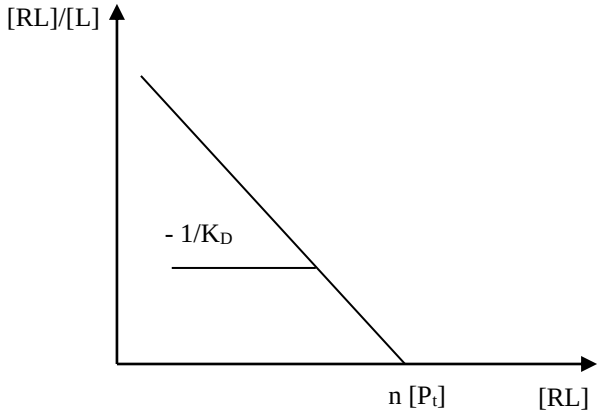
$$[L] / [RL] = K_D / (n [P_t] – [RL])$$

$$[RL] / [L] = (n [P_t] – [RL]) / K_D$$

$$\mathbf{[RL] / [L] = - [RL] /K_D + (n [P_t] / K_D)}$$

Therefore, SCATCHARD's representation consists of graphing [RL]/[L] as a function of [RL]. The graph is a straight line with a slope of ($-K_A$) or ($-1/K_D$) that intersects the x-axis at (n $[P_t]$).

Since $[P_t]$ is known, we can deduce (n).

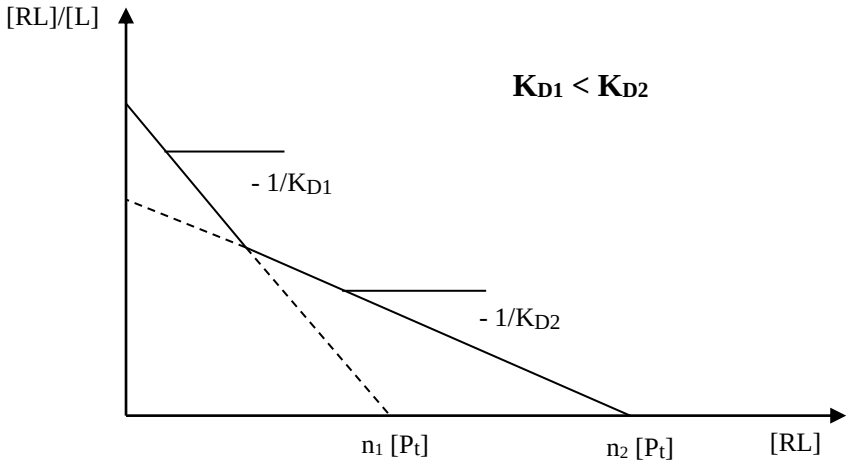


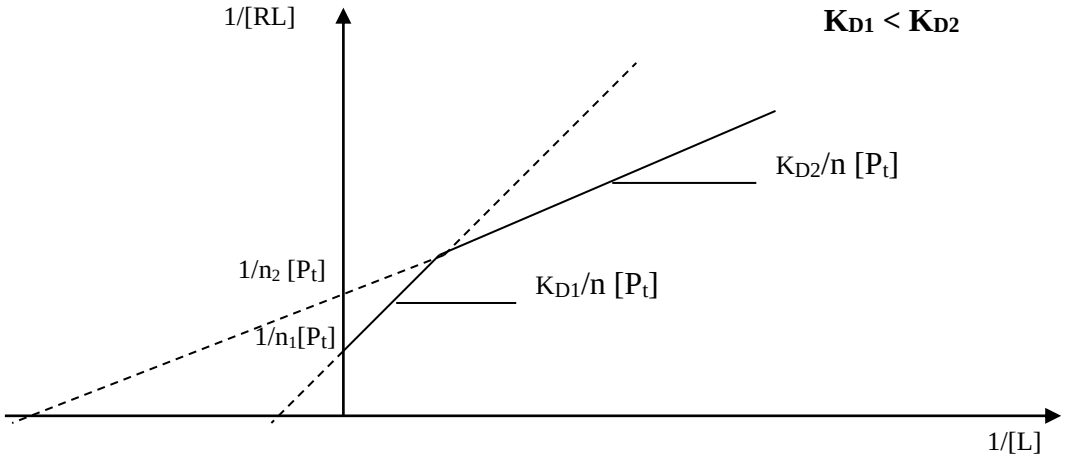
3. 2. 2. Independent and non-equivalent sites :

When sites are not equivalent (having different affinities for the ligand), site classes are defined; each class groups together sites with the same dissociation constant (K_D). Consequently, the SCATCHARD and KLÖTZ representations are no longer linear.

Example :

For a protein that has two classes of sites, and therefore two different dissociation constants ($K_{D1} \neq K_{D2}$), in other words, two different affinities, we obtain two straight line segments with two different slopes (the greater the slope, the greater the affinity).





4. Experimental approaches :

The determinationon of the number of binding sites (n) generally requires the determination of the bound ligand concentration [RL] and that of the free ligand [L]. These concentrations are measured by :

4.1. Physical separation :

Ultrafiltration

- ✓ It uses the property of certain filters that absorb proteins while the free ligand is not retained.
- ✓ The protein-ligand mixture is filtered through supports such as nitrocellulose or semi-permeable membranes.
- ✓ The proportions of bound ligand retained on the filter and free ligand in the filtrate can then be measured.

Ultracentrifugation

- ✓ This method involves subjecting the protein-ligand mixture to a gravitational field.
- ✓ After a suitable period of time, the supernatant contains the free ligand, while the pellet contains the ligand bound to the protein.
- ✓ Ultracentrifugation is a laboratory technique that uses extremely high-speed centrifugation to separate components of a mixture based on their size, shape, and density. By spinning samples at speeds up to 1,000,000 x g, it generates a strong centrifugal force that causes particles to sediment, or settle, at different rates.

Dialysis at equilibrium

- ✓ This dialysis is performed through a semi-permeable membrane with relatively small pores, so that the membrane is permeable to small ligand molecules but remains impermeable to protein.
- ✓ Membranes made of cellulose, cellophane, or collodion are mainly used.

- ✓ When equilibrium is achieved, the concentration of free ligand inside and outside the bag is virtually the same.

Molecular size exclusion chromatography :

- ✓ Chromatography is a laboratory technique used to separate a mixture into its individual components. It works by passing the mixture through a stationary phase, which is a solid or liquid, using a mobile phase (a liquid or gas). The different components of the mixture separate because they travel at different speeds, depending on their affinity for the stationary and mobile phases.
- ✓ In this technique, molecular sieves such as Sephadex are used to separate the free ligand from the bound ligand.
- ✓ These sieves delay the elution of small molecules and allow larger molecules to pass through.
- ✓ The ligand must have a characteristic (radioactivity or absorption band) that allows its total concentration in the eluate to be measured.

4.2. *Indirect spectrophotometric measurement :*

Fluorescence.

- ✓ Fluorescence methods can be used when the ligand exhibits fluorescence that is sufficiently altered upon binding to the protein.
- ✓ In some cases, it is possible to use the fluorescence of the protein when the binding of the ligand causes a significant attenuation of this fluorescence.