

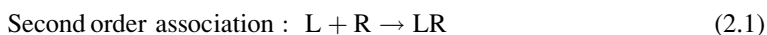
## Chapter 2

# The Basics

Chemical reactions of the first and second order are the basis of all binding kinetics, binding equilibria and enzyme kinetics. Kinetic experiments must be calculated from a set of differential equations, whereas equilibrium binding studies can be calculated from a set of ordinary equations. Chapter 2 provides guidelines to write such equations for any reaction scheme. This is independent of the solution, be it analytical or numerical.

### 2.1 Equations for First and Second Order Reactions

When a ligand L binds to a receptor R (2.1), the probability of interaction is proportional to both concentrations. The rate of product formation  $d[LR]/dt$  therefore is the product of these concentrations times a constant, which is called the rate constant  $k$ . Rate constants are denoted as lower case letters.



$$\text{Second order rates : } d[LR]/dt = k_1 \cdot [L] \cdot [R] \quad (2.2)$$

$$d[L]/dt = -k_1 \cdot [L] \cdot [R] \quad (2.3)$$

$$d[R]/dt = -k_1 \cdot [L] \cdot [R] \quad (2.4)$$

These are differential equations. They have to be written for the concentration changes of all components of the reaction, for L, R and LR. Note that we follow the convention and write concentrations in square brackets. This convention cannot be upheld in computer code, where a square bracket cannot be part of a variable name. In octave and MATLAB, square brackets define a vector or matrix.

Once the complex LR has been formed, it may or may not dissociate. The dissociation rate is proportional to the concentration of the complex.



$$\text{First order rates : } d[LR]/dt = -k_{-1} \cdot [LR] \quad (2.6)$$

$$d[L]/dt = k_{-1} \cdot [LR] \quad (2.7)$$

$$d[R]/dt = k_{-1} \cdot [LR] \quad (2.8)$$

The negative sign in (2.6) indicates that the concentration of LR is decreased upon the decay of the complex. Reaction schemes (2.1) and (2.5) together with the basic reaction rates (2.2)–(2.4) and (2.6)–(2.8) contain all the physics required to understand this textbook. Complex biochemical reactions result from bimolecular association and monomolecular dissociation and combinations of these steps.

The above differential equations have been solved analytically, but let us use this example to understand numerical solutions of differential equations: Mathematically, a differential quotient  $dx/dt$  is the limiting value of the difference quotient  $\Delta x/\Delta t$  for infinitesimal small differences. Computer programs use a practical version of this definition: Calculate the differences  $\Delta x$  of all concentrations for a small time interval  $\Delta t$ , add these differences to the original values and do the calculations for the next time interval. Repeat these steps until the time range of interest is covered. That is all. For ordinary differential equations (they can be written as  $dx/dt = f(x, t)$ , like all combinations of first and second order reactions) octave provides the function `lsode`, a general procedure to solve them. The function `lsode` will be covered in Chap. 6, but let us discuss the general principle with the simple example of a reversible reaction:



Reversible binding (2.9) leads to the formation of the complex LR and to a corresponding decrease of L and R. The difference quotients are:

$$\Delta[L]/\Delta t = -k_1 \cdot [L] \cdot [R] + k_{-1} \cdot [LR] \quad (2.10)$$

$$\Delta[R]/\Delta t = -k_1 \cdot [L] \cdot [R] + k_{-1} \cdot [LR] \quad (2.11)$$

$$\Delta[LR]/\Delta t = k_1 \cdot [L] \cdot [R] - k_{-1} \cdot [LR] \quad (2.12)$$

When all rate constants and all initial concentrations are known, the concentration changes  $\Delta[LR]$ ,  $\Delta[L]$  and  $\Delta[R]$  can be calculated from (2.10)–(2.12) for any given time interval  $\Delta t$ .

Let us assume that the experiment simply consists of mixing receptor and ligand to total concentrations  $R_0$  and  $L_0$ , respectively. At time zero, the concentrations are  $[R] = R_0$ ,  $[L] = L_0$ ,  $[LR] = 0$ . After the small time interval  $\Delta t$ , these concentrations are  $[R] = R_0 + \Delta[R]$ ,  $[L] = L_0 + \Delta[L]$  and  $[LR] = 0 + \Delta[LR]$ .

For the next time interval  $\Delta t$  these new concentrations have to be inserted in (2.10)–(2.12) in order to compute the new concentration changes, and so forth. These are very basic repetitive operations, ideally suited for a computer.

*Equilibrium* is reached for reversible reactions (2.9) when the dissociation rate (1.6) is equal to association rate (2.2)

$$k_1 \cdot [L] \cdot [R] = k_{-1} \cdot [LR] \quad (2.13)$$

This leads to (2.14) with the equilibrium dissociation constant  $K_{D1}$ . Equilibrium constants  $K_1 = k_1/k_{-1}$  and equilibrium dissociation constants  $K_{D1} = k_{-1}/k_1$  are denoted with uppercase letters. In life science and in this textbook, equilibrium dissociation constants  $K_D$  are used rather than equilibrium constants  $K$ . They correspond to the ligand concentration where half of the binding sites for this  $K_D$  are occupied.

$$K_{D1} = k_{-1}/k_1 = [L] \cdot [R]/[LR] \quad (2.14)$$

$$[LR] = [L] \cdot [R]/K_{D1} \quad (2.15)$$

Bound ligand and free ligand concentrations must add up to the total ligand concentration  $L_0$ . The same must hold for all components of bound and free receptors which add up to the total receptor concentration  $R_0$ :

$$R_0 = [R] + [L] \cdot [R]/K_D \quad (2.16)$$

$$L_0 = [L] + [L] \cdot [R]/K_D \quad (2.17)$$

Equations (2.16) and (2.17) are two equations with the two unknowns  $[L]$  and  $[R]$ . They follow the pattern: “The total concentration of each molecule must be the sum of free and bound concentrations”. This pattern leads to  $n$  equations of  $n$  unknowns. Even the most complex equilibria can be calculated from these equations. Analytical solutions for such general equations involving numerous complexes and numerous ligands may be difficult or impossible to find, but numerical solutions are relatively easy with the help of a suitable algorithm. Again, as has been discussed for differential equations, the main task for the scientist lies in writing the equations.

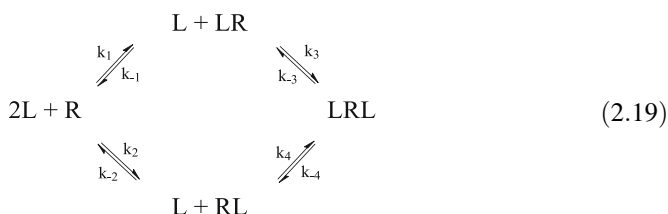
## 2.2 Equilibrium Binding to Two Sites

The binding of ligands to two sites of a receptor is often described with reaction scheme (2.18), where a ligand may bind to two sites. Only one singly bound complex  $LR$  and one doubly bound  $L_2R$  are considered in this scheme. The scheme

can be extended to incorporate more binding sites, always with only one complex and one equilibrium dissociation constant for each sequential binding step



Scheme (2.18) is ideally suited for the analysis of equilibrium binding studies, since it involves a minimal number of constants. It is, however, not a plausible scheme. Generally, one would expect the ligand to have a choice for binding to one or to the other site, before the second ligand binds to the non-occupied site. Such a mechanism of accessible sites is shown in scheme (2.19) where the first ligand may bind to the “left” (LR) or “right” (RL) site, before a second ligand leads to the formation of the fully occupied receptor LRL.



Reaction scheme (2.19) has sometimes been called “diamond model” because of its shape. It illustrates the most general model for two site binding. Sites are considered to be independent when the ligand bound to one site has the same affinity independent of the occupation of the other site. This statement translates to  $K_{D1} = K_{D4}$  and  $K_{D2} = K_{D3}$ . The sites are equivalent when their affinity is the same, i.e.  $K_{D1} = K_{D2}$ . For equivalent sites, binding is regarded to be cooperative, when  $K_{D1} > K_{D3}$ , so that the second ligand binds with a higher affinity. For  $K_{D1} < K_{D3}$  and equivalent sites, the binding mechanism is called anticooperative.

One should note that reaction scheme (2.19) shows a coupled equilibrium and that the affinity of the ternary complex LRL must be independent of the path by which it was formed.

$$\text{Upperpath : } [LR] = [L] \cdot [R]/K_{D1} \quad [LRL] = [LR] \cdot [L]/K_{D3} \quad (2.20)$$

$$\text{Lowerpath : } [RL] = [L] \cdot [R]/K_{D2} \quad [LRL] = [RL] \cdot [L]/K_{D4} \quad (2.21)$$

If we combine all four equations, we get

$$[L] \cdot [L] \cdot [R]/(K_{D1} \cdot K_{D3}) = [L] \cdot [L] \cdot [R]/(K_{D2} \cdot K_{D4}) \quad (2.22)$$

And thus

$$K_{D1} \cdot K_{D3} = K_{D2} \cdot K_{D4} \quad (2.23)$$

One equilibrium dissociation constant of a coupled equilibrium can therefore be calculated from the other ones. This principle holds for all coupled equilibria, since the affinity of a complex must be independent of the path by which it was formed. The concentrations of the complexes LR, RL and LRL, written in (2.20)–(2.22) are functions of free concentrations [L] and [R]. The total concentrations R0 and L0 must therefore be equal to

$$R0 = [R] + [L] \cdot [R]/K_D1 + [L] \cdot [R]/K_D2 + [L] \cdot [L] \cdot [R]/(K_D1 \cdot K_D3) \quad (2.24)$$

$$L0 = [L] + [L] \cdot [R]/K_D1 + [L] \cdot [R]/K_D2 + 2 \cdot [L] \cdot [L] \cdot [R]/(K_D1 \cdot K_D3) \quad (2.25)$$

Note that the complex LRL contains two bound ligand molecules. This is taken into account by the factor 2 in (2.25). For reaction scheme (2.18), the corresponding sets of equations are given in (2.26) and (2.27):

$$R0 = [R] + [L] \cdot [R]/K_D1 + [L] \cdot [L] \cdot [R]/(K_D1 \cdot K_D2) \quad (2.26)$$

$$L0 = [L] + [L] \cdot [R]/K_D1 + 2 \cdot [L] \cdot [L] \cdot [R]/(K_D1 \cdot K_D2) \quad (2.27)$$

Note that  $K_{D2}$  in reaction scheme (2.18) indicates the occupation of the second ligand to form the fully saturated complex LRL, whereas  $K_{D2}$  in reaction scheme (2.19) denotes the binding of one ligand to an independent second site. Equations (2.24) and (2.25) can be re-written as (2.28) and (2.29).

$$R0 = [R] + [L] \cdot [R] \cdot (1/K_D1 + 1/K_D2) + [L] \cdot [L] \cdot [R]/(K_D1 \cdot K_D3) \quad (2.28)$$

$$L0 = [L] + [L] \cdot [R] \cdot (1/K_D1 + 1/K_D2) + 2 \cdot [L] \cdot [L] \cdot [R]/(K_D1 \cdot K_D3) \quad (2.29)$$

They are identical when  $1/K_{D1}$  for scheme (2.18) equals  $(1/K_{D1} + 1/K_{D2})$  from scheme (2.19) and  $K_{D1} \cdot K_{D2}$  from scheme (2.18) equals  $K_{D1} \cdot K_{D3}$  from scheme (2.19).

Equilibrium binding studies therefore cannot distinguish between the sequential scheme (2.18) and a more plausible scheme of accessible sites (2.19). It will be shown in Sect. 6.5 that a sequential binding mechanism (2.18) can be identified with a kinetic “chase” experiment. But only a *kinetic* experiment with two different ligands allows the distinction between first and second bound ligand.

## 2.3 Equilibrium Binding to Any Number of Sites

These conclusions can be generalized: Equilibrium binding studies can only determine one effective affinity for each occupation of sites, no matter how many individual complexes can be identified for each monoliganded, diliganded,

triliganded, etc. occupation. If we know the intrinsic affinities of each individual binding site at each step, we can compute the simplified affinities of a sequential scheme. This reasoning does not work in the opposite direction: Since equilibrium binding studies can only determine one effective equilibrium dissociation constant for the different occupation of sites, intrinsic equilibrium dissociation constants for the different sites cannot be deduced without further assumptions.

Only if we assume that affinities of all  $n$  individual binding sites are equivalent, can intrinsic equilibrium dissociation constants  $K_{D,i}^{\text{intrinsic}}$  for the  $i$ th steps be calculated from the effective (sequential) equilibrium dissociation constants (2.30).

$$K_{D,i}^{\text{intrinsic}} = ((n - i + 1)/i) \cdot K_{D,i}^{\text{sequential}} \quad (2.30)$$

As an example, let us assume two step sequential binding sites with  $K_{D,1}^{\text{sequential}} = K_{D,2}^{\text{sequential}} = 10 \mu\text{M}$  according to reaction scheme (2.18). These equilibrium dissociation constants of scheme (2.18) translate to  $K_{D,1}^{\text{intrinsic}} (=K_{D,2}^{\text{intrinsic}}) = 20 \mu\text{M}$  and  $K_{D,3}^{\text{intrinsic}} (=K_{D,4}^{\text{intrinsic}}) = 5 \mu\text{M}$  of reaction scheme (2.19). Therefore, identical equilibrium dissociation constants for each step of a sequential mechanism (like (2.18)) translate into cooperative binding when intrinsic equilibrium dissociation constants of accessible sites are computed.

Calculating equilibrium binding curves from any given reaction scheme is quite straight-forward. One simply writes one equation (2.31) for each molecule of the reaction

$$\text{Total concentration} = \text{free concentration} + \text{bound concentration} \quad (2.31)$$

This corresponds to (2.24) and (2.25). The bound concentration is the sum of all molecules bound to all complexes. The set of (2.31) can be solved numerically. These equations need not be linear, and there may be more than one solution. A reasonable initial estimate ensures that the right solution is found. This is discussed in Chap. 5.

## 2.4 Writing Differential Equations for Two Site Binding

Differential equations can readily be solved with numerical methods, but they have to be set up first. For example, analyzing reaction scheme (2.18) involves the calculation of four concentrations, namely  $[R]$ ,  $[L]$ ,  $[LR]$ , and  $[L_2R]$ . The four differential equations for the four concentration changes are given in (2.32)–(2.35)

$$d[R]/dt = -k_1 \cdot [R] \cdot [L] + k_{-1} \cdot [LR] \quad (2.32)$$

$$d[L]/dt = -k_1 \cdot [R] \cdot [L] + k_{-1} \cdot [LR] - k_2 \cdot [LR] \cdot [L] + k_{-2} \cdot [L_2R] \quad (2.33)$$

$$d[LR]/dt = k_1 \cdot [R] \cdot [L] - k_{-1} \cdot [LR] - k_2 \cdot [LR] \cdot [L] + k_{-2} \cdot [L_2R] \quad (2.34)$$

$$d[L_2R]/dt = k_2 \cdot [LR] \cdot [L] - k_{-2} \cdot [L_2R] \quad (2.35)$$

Note the algebraic signs. Since all concentrations and rate constants are positive, a decrease in concentration, that is a negative rate, always is indicated by a negative sign. Note (2.34), where LR is decreased by the dissociation part of reaction 1 and the association part of reaction 2. Any second order reaction involves three components of the reactions. Therefore, reaction 1 with  $k_1$  and  $k_{-1}$  affects the concentration changes of R, L and LR in (2.32), (2.33) and (2.34). Likewise, reaction 2 with  $k_2$  and  $k_{-2}$  involves the components L, LR and  $L_2R$  and thus affects (2.33), (2.34) and (2.35). For more complex schemes, such a check that a given rate constant must be involved in three (or two, for reversible first order) reactions helps in debugging a program. One can use the “find” function included in any text editor to make sure that each rate constant of a reversible reaction of the second order appears three times.

Differential equations for reaction scheme (2.19) require the calculation of five concentrations, namely [R], [L], [LR], [RL], and [LRL]. This is shown in equations (2.36)–(2.40):

$$d[R]/dt = -k_1 \cdot [R] \cdot [L] + k_{-1} \cdot [LR] - k_2 \cdot [R] \cdot [L] + k_{-2} \cdot [RL] \quad (2.36)$$

$$d[L]/dt = -k_1 \cdot [R] \cdot [L] + k_{-1} \cdot [LR] - k_3 \cdot [LR] \cdot [L] + k_{-3} \cdot [LRL] \\ - k_2 \cdot [R] \cdot [L] + k_{-2} \cdot [RL] - k_4 \cdot [RL] \cdot [L] + k_{-4} \cdot [LRL] \quad (2.37)$$

$$d[LR]/dt = k_1 \cdot [R] \cdot [L] - k_{-1} \cdot [LR] - k_3 \cdot [LR] \cdot [L] + k_{-3} \cdot [LRL] \quad (2.38)$$

$$d[RL]/dt = k_2 \cdot [R] \cdot [L] - k_{-2} \cdot [RL] - k_4 \cdot [RL] \cdot [L] + k_{-4} \cdot [LRL] \quad (2.39)$$

$$d[LRL]/dt = k_3 \cdot [LR] \cdot [L] - k_{-3} \cdot [LRL] + k_4 \cdot [RL] \cdot [L] - k_{-4} \cdot [LRL] \quad (2.40)$$

The more complex a reaction scheme becomes, the more and longer differential equations have to be computed. Each reversible reaction corresponds to two products of rate constant and component of the reactions. Note that the free ligand L is involved in all four reactions, so that there are eight products in (2.37). There are two reactions involving the free receptor R in reaction scheme (2.19), so that there are four products in (2.36). Likewise, two pathways for the dissociation of the complex LRL are reflected in four products in (2.40).

One important restriction must be considered: Reaction scheme (2.19) contains a closed loop which corresponds to coupled equilibria calculated in (2.23). This reduces the number of independent rate constants from 8 (4 reversible reactions) to 7. Equation (2.23), written for rate constants instead of equilibrium constants, translates into:

$$k_1 \cdot k_3 \cdot k_{-2} \cdot k_{-4} = k_2 \cdot k_4 \cdot k_{-1} \cdot k_{-3} \quad (2.41)$$

Again (2.41) ensures consistency, just like (2.23). It may be interpreted as a rule for circular reactions: The product of rate constants in one (clockwise) direction of reaction scheme (2.19) must be the same as the product of rate constants in the other (counterclockwise) direction. One of the rate constants in a closed loop therefore can be calculated from the other ones.

## 2.5 Writing Differential Equations for Any Reaction Scheme

Writing differential equations for a complex reaction scheme may look complicated, but it does not require much fantasy. First, one counts all complexes and free ligands. Then one has to write one, and possibly a long one, differential equation for the concentration change of each of these components. For reversible reactions in a fixed volume in solution, these concentration changes can only be expected from second or first order reactions. Each second order reaction has to be considered in the concentration changes of all three components of the reaction. There may be first order conformational changes which involve only two components (one for each conformation) or first order decay which only involves one component, provided the product is irrelevant and the decay is not reversible. Closed circles in reaction schemes have to fulfill the criterion of (2.41), namely that the products of all rate constants in one direction must be the same as the products of rate constants in the other direction.

That is all. But when differential equations are written as part of a program code, no typing error is allowed. It helps to use a “find” function in a program editor and look for all the rate constants individually. Any rate constant involved in a bimolecular reversible reaction must appear thrice. Whenever a given concentration appears in the differential equation of this concentration, the algebraic sign in front of the accompanying rate constant must be negative. For reversible reactions, the number of products in each differential equation must be even. Those little controls may help.

## 2.6 Analytical and Numerical Solutions

Only for the simplest cases, the sets of equations described above can be solved analytically. But when an analytical solution is found, it is precise and reliable for all feasible concentrations. Finding analytical solutions needs a lot of effort, but calculating them can be done from one formula with simple spread sheets or pocket calculators. Some of the most important analytical solutions are covered in Chap. 3.

Numerical methods are different. They are well established, but they depend on a computer and a program to run them with. The algorithms are based on approximations. The results are the same within reasonable errors, but they are not identical to analytical formulas. Figure 5.2 illustrates this with one example. All calculations done by computers are limited by the precision of the stored variables. When small differences of large numbers approach the precision of those large numbers, they become unreliable. Octave and MATLAB typically issue warnings when the internal precision is not sufficient. In general, whenever stochastic results are computed with numerical methods, one should repeat the calculations with other parameters (concentrations or rate constants). Use a computer, but keep checking it and do not develop unconfined trust!

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