4.20: Thermodynamic Factors

The equilibrium constant K was defined in Equation (4.3) in terms of the activity a_i of component i. The a_i may be expressed as a function of concentration as

$$a_i = \gamma_i [i], \tag{4.7}$$

where for species i, γ_i is its activity coefficient and [i] is its concentration (strictly molality, but usually as molarity in mol L^{-1}). At infinite dilution $\gamma_i = 1$. Provided that the charge and size of species M and MO_2 are similar and that O_2 forms an ideal solution, then the activities of Equation (4.3) may be approximated by concentrations to give the expression

$$K_c = \frac{[MO_2]}{[M][O_2]}. \tag{4.8}$$

However, Equation (4.8) does not permit a direct comparison of the oxygen-binding behavior of one species in some solvent with that of a second in some other solvent. First, for a given partial pressure of dioxygen, the concentration of O_2 in the solution varies considerably with temperature and from one solvent to another. Second, reliable measurements of oxygen solubilities are not always available, and it is only relatively recently that oxygen electrodes have been developed to measure directly oxygen concentrations (strictly, activities). However, oxygen-binding measurements are normally made with a solution of M in equilibrium with gaseous dioxygen. At equilibrium the molar Gibbs' free energies (chemical potentials) of the dissolved and gaseous dioxygen are identical—if they are not, gaseous O_2 would dissolve, or dissolved O_2 would be released. Thus the solvent-dependent quantity $[O_2]$ in Equation (4.8) may be replaced by the solvent-independent quantity $P(O_2)$, the partial pressure of dioxygen. Under almost all experimental conditions the quantity $P(O_2)$ is a very good approximation to the gas-phase activity (fugacity) of dioxygen; hence we obtain for the equilibrium constant*

$$K_p = \frac{[MO_2]}{[M]P(O_2)}. \tag{4.9}$$

* There has been considerable discussion as to whether K_c (4.8) or K_p (4.9) should be used to compare dioxygen binding under different solvent conditions[21-23] We believe that the latter is more appropriate, since for a system al equilibrium, the chemical potential of gaseous O_2 must be identical with that of dissolved O_2[19] On the other hand, the concentration of O_2 varies from one solvent to another.

It is very convenient to express the affinity as the partial pressure of dioxygen required for half-saturation of the species M, $P_{1/2}(O_2)$. Under such conditions, [M] = $[MO_2]$, one obtains

$$P_{1/2}(O_2) = 1/K_p, \tag{4.10}$$

where $P_{1/2}(O_2)$ is usually given in Torr or mm Hg.* As will be detailed shortly, values for $P_{1/2}(O_2)$ are typically in the range 0.5 to 40 Torr.

The dioxygen affinity is composed of enthalpic (ΔH) and entropic (ΔS) components, with

$$\Delta G^o = -RTlnK = \Delta H^o - T\Delta S^o. \tag{4.11}$$

Within a family of oxygen carriers the values of ΔS^o and ΔH° are usually similar. Large deviations (such as a change of sign) are therefore indicative of a change in the nature of the oxygen-binding process.

* Many authors use the symbol P_{50} (corresponding to 50% saturation) for $P_{1/2}$.

a. Non-cooperative Dioxygen Binding

If the oxygen-binding sites M are mutually independent and noninteracting, as in moderately dilute solutions of monomeric molecules, then the concentration of species MO_2 as a function of the partial pressure of O_2 is generally well fit by a Langmuir isotherm.[20] Here a plot of the fractional saturation of dioxygen binding sites, θ, where

$$\theta = \frac{[MO_2]}{[M]+[MO_2]} = \frac{K_p P(O_2)}{1+K_p P(O_2)} \tag{4.12}$$

versus $P(O_2)$ gives the hyberbolic curve labeled "non-cooperative" in Figure 4.4A.[9] Alternatively,[24] a plot of log (θ/(1 - θ)) versus log ($P(O_2)$), the so-called "Hill plot," gives a straight line with a slope of unity and an intercept of -log $P_{1/2}(O_2)$ (Figure 4.4B). A differential form is shown as the dotted line in Figure 4.4C. Such binding, where the dioxygen sites are independent of each other, is termed *non-cooperative*.

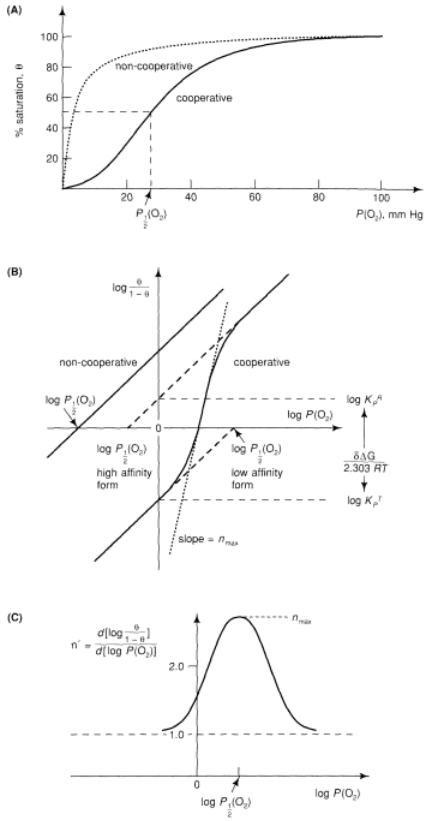

Figure 4.4 - Cooperative and non-cooperative binding of dioxygen:[9] (A) Binding curves; (B) Hill plot of binding curves; (C) First derivative (slope) of the Hill plots.

b. Cooperative Dioxygen Binding

Many dioxygen-binding proteins are not independent monomers, with only one dioxygen-binding site, but oligomeric species with the protein comprising two or more similar subunits. The subunits may be held together by van der Waals' forces or by stronger interactions, such as hydrogen bonds or salt bridges, or even by covalent bonds. For example, most mammalian hemoglobins are tetramers, consisting of two pairs $[\alpha\beta]_2$ of myoglobin-like subunits denoted as α and β. Either none, one, two, three, or all four sites may be occupied by dioxygen. This situation is illustrated schematically in Figure 4.5, which also shows the statistical weighting of each level of saturation, treating the α and β subunits as identical. Thus the binding or release of dioxygen at one site *may* affect the affinity and kinetics of ligand binding and release at a neighboring site. As a result, the saturation curve becomes sigmoidal in shape, as illustrated in Figure 4.4A. The dioxygen binding is *cooperative*. When cooperativity is positive, the affinity of a vacant site is increased by occupancy of an adjacent one.

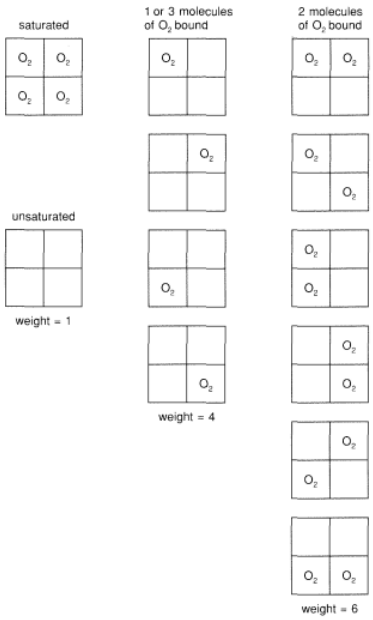

Figure 4.5 - Diagram of tetrameric hemoglobin, showing statistical weights of different saturations (see text).

This behavior, where the binding of one molecule influences the binding of successive molecules *of the same kind*, is referred to as a *homotropic allosteric* interaction. A *heterotropic allosteric* interaction occurs when the interaction with the protein of a second *unlike* molecule, for instance, an organic polyphosphate for human hemoglobins, influences the binding of the first molecule (e.g., dioxygen). Such molecules are often termed *allosteric effectors*. A commonly observed heterotropic allosteric interaction is the Bohr effect, named after the biologist Christian Bohr, father of physicist Niels Bohr. This effect, which relates the change in partial pressure of O_2 to a change in pH at constant saturation of binding sites (θ), is related thermodynamically to the Haldane effect, which relates the number of protons released ($\#H^+$) with a change in θ at constant pH (Equation 4.13). A very large Bohr effect, where O_2 affinity decreases sharply with pH, is often called the Root effect.[25a] It is physiologically important for fish such as trout, probably in maintaining buoyancy, but its molecular basis in trout hemoglobin IV remains to be discovered.[25b]

$$\left[\frac{\partial(\#H^+)}{\partial\theta}\right]_{pH} = \left[\frac{\partial(logP(O_2))}{\partial pH}\right]_{\theta} \tag{4.13}$$

The degree of cooperativity can be characterized in a number of ways. By means of a Hill plot of log (θ/(1 - θ)) versus log ($P(O_2)$), the limiting slopes (which should be unity) at high O_2 pressure and low O_2 pressure may be extrapolated as shown in Figure 4.4B to log (θ/(1 - θ)) = 0, where θ = 0.5. Two limiting values for $P_{1/2}(O_2)$ are obtained, one characterizing the regime of high partial pressure of dioxygen, where the O_2 affinity is high (for the case illustrated of positive cooperativity). The other $P_{1/2}(O_2)$ value characterizes the regime of low partial pressure of dioxygen, where affinity is relatively low. This difference in affinities can be converted into a difference between the free-energy change upon O_2 binding in the low-affinity state (K_p^T) and the high-affinity state (K_p^R) [the designations T and R will be described in subsection d]:

$$\delta\Delta G^o = -RTln\left(\frac{K_p^T}{K_p^R}\right). \tag{4.14}$$

A second way to characterize cooperativity involves fitting the oxygen-binding data at intermediate saturation (0.2 < θ < 0.8)—that is, about the inflection point in a Hill plot—to the Hill equation

$$\frac{\theta}{1-\theta} = K_p P^n(O_2) \tag{4.20.1}$$

or

$$log\left(\frac{\theta}{1-\theta}\right) = -log(P_{1/2}(O_2)) + nlog(P(O_2)). \tag{4.15}$$

The Hill coefficient (n) is an empirical coefficient that has a value of unity for non-cooperative binding, where Equation (4.15) reduces to the Langmuir isotherm, Equation (4.12). Any number greater than unity indicates positive cooperativity. If O_2 binding is an all-or-nothing affair, where dioxygen binding sites are either all occupied or all vacant, n equals the number of subunits in the molecule. The fit is only approximate, since the Hill plot is only approximately linear about the inflection point, as may be seen in Figure 4.4B. A more precise value of n may be obtained by plotting the slope in the Hill plot (n') as a function

$$n' = \frac{d\left[log\left(\frac{\theta}{1-\theta}\right)\right]}{d[log(P(O_2))]} \tag{4.16}$$

of log ($P(O_2)$) (Figure 4.4C). The maximum value of n' is taken as the Hill coefficient n.[9] Note that the maximum in this first-derivative plot of the binding curve will occur at $P_{1/2}(O_2)$ only if the Hill plot is symmetric about its inflection point. For tetrameric hemoglobins, a maximum Hill coefficient of around 3.0 is seen, and for hemocyanins n may be as high as 9. These values, like $P_{1/2}(O_2)$ values, are sensitive to the nature and concentrations of allosteric effectors.

c. Benefits of Cooperative Ligand Binding

In general, oxygen-carrier proteins, being oligomeric, coordinate dioxygen cooperatively, whereas oxygen-storage proteins, being monomeric, do not. Oligomerization and cooperative binding confer enormous physiological benefits to an organism. The first benefit derives directly from oligomerization. Oxygen carriers either form small oligomers that are encapsulated into cells or erythrocytes (such hemoglobins are referred to as intracellular hemoglobins) or associate into large oligomers of 100 or more subunits. Such encapsulation and association reduce by orders of magnitude the number of independent particles in the blood, with consequent reductions in the osmotic pressure of the solution and in strain on vascular membranes.

The second benefit derives from cooperative binding of ligands and the abilities of heterotropic allosteric effectors to optimize exquisitely the oxygen-binding behavior in response to the external and internal environment. The situation is illustrated in general terms in Figure 4.6.[9] Most organisms that require O_2 live in an environment where the activity of O_2 corresponds to about 21 percent of an atmosphere, that is, to about 160 Torr, although usually the effective availability, because of incomplete exchange of gases in the lungs, for example, is around 100 Torr. The concentration of O_2 in vertebrate tissues at rest is equivalent to a partial pressure of about 35-40 Torr dioxygen; lower values obtain at times of exertion. Now consider a noncooperative oxygen binder with an affinity expressed as $P_{1/2}(O_2)$ of 60 Torr (Figure 4.6, curve a). Then, at 100 Torr the fractional saturation θ is 0.625. In other words, in a realm of high O_2 availability, only 62.5 percent of the oxygen-binding capacity is used, which is not particularly efficient if the organism wished to climb Mt. Everest, where the partial pressure of O_2 is less than half that at sea level. In the tissues, where $P(O_2)$ = 40 Torr, the fractional saturation is about 40 percent. Thus, only about one third of the coordinated dioxygen is released to the tissues, and total effciency is only 22.5 percent. Consider now a noncooperative oxygen carrier with a much higher affinity, $P_{1/2}(O_2)$ = 1.0 Torr (Figure 4.6, curve b). If we assume the same ambient pressure of O_2 in the tissues, the fractional saturation is 97.6 percent. Note that at 100 Torr of O_2 the carrier is 99.0 percent saturated. In other words, only about 1.4 percent of the available oxygen is delivered.

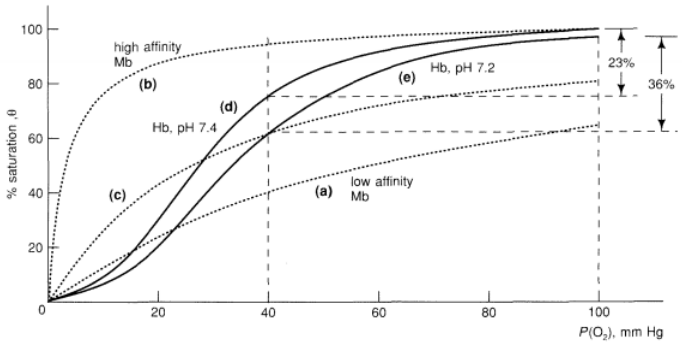

Figure 4.6 - Physiological benefits of cooperativity and heterotropic allosteric effectors.[9]

With an oligomeric protein that binds dioxygen cooperatively, the problem of inefficient and inflexible oxygen delivery disappears. For example, the tetrameric protein hemoglobin has a mean affinity for O_2 of $P_{1/2}(O_2) \approx 26$ Torr at 37 °C and pH 7.4. If hemoglobin bound O_2 noncooperatively, then the hyberbolic binding curve (c) in Figure 4.6 would represent the O_2 binding. Instead, the observed binding follows curve (d). Since the partial pressure of dioxygen in the lungs and arterial blood of vertebrates

is around 100 Torr, but in the tissues and venous blood it is around 40 Torr, then at these pressures a typical myoglobin ($P_{1/2}(O_2)$ = 1 Torr) remains effectively saturated. On the other hand, about 25 percent of the available dioxygen can be delivered, even in the absence of myoglobin. With venous blood remaining 75 percent oxygenated, hemoglobin has substantial capacity to deliver more O_2 at times of exertion or stress when $P(O_2)$ in the tissues falls below 40 Torr.

The net result is that whole blood, which contains about 15 g of hemoglobin per 100 mL, can carry the equivalent of 20 mL of O_2 (at 760 Torr) per 100 mL, whereas blood plasma (no hemoglobin) has a carrying capacity of only 0.3 mL of O_2 per 100 mL.[9]

Oxygen binding *in vivo* is modulated by allosteric effectors that through interaction with the protein change the affinity and degree of cooperativity. For hemoglobin A (adult human hemoglobin), naturally occurring allosteric effectors include the proton, carbon dioxide, and 2,3-diphosphoglycerate (2,3-DPG). Increasing concentrations of these species progressively lower the affinity of free hemoglobin A, thereby enhancing the release of coordinated O_2 (Figure 4.6, curve e). For example, 2,3-DPG is part of a subtle mechanism by which dioxygen is transferred from mother to fetus across the placenta. The subunits comprising fetal hemoglobin and adult hemoglobin are slightly different. In the absence of allosteric effectors (referred to as stripped hemoglobin), the oxygen-binding curves are identical. However, 2,3-DPG binds less strongly to fetal hemoglobin than to adult hemoglobin. Thus fetal hemoglobin has a slightly higher affinity for dioxygen, thereby enabling dioxygen to be transferred. The proton and carbon dioxide are part of a short-term feedback mechanism. When O_2 consumption outpaces O_2 delivery, glucose is incompletely oxidized to lactic acid (instead of CO_2). The lactic acid produced lowers the pH, and O_2 release from oxyhemoglobin is stimulated (Figure 4.6, curve e). The CO_2 produced in respiration forms carbamates with the amino terminals, preferentially of deoxy hemoglobin.

$$R-NH_2+CO_2 \rightleftharpoons R-NH-COO^- + H^+ \tag{4.20.2}$$

Thus hemoglobin not only delivers O_2 but also facilitates removal of CO_2 to the lungs or gills, where CO_2 is exhaled.

d. Models for Cooperativity

The binding of Oz to hemoglobin can be described as four successive equilibria:

$$Hb + O_2 \overset{K^{(1)}}{\rightleftharpoons} Hb(O_2) \qquad P_{1/2}^{(1)}(O_2) = 123[46]Torr \tag{4.20.3}$$

$$Hb(O_2)_1 + O_2 \overset{K^{(2)}}{\rightleftharpoons} Hb(O_2)_2 \qquad P_{1/2}^{(2)}(O_2) = 30[16]Torr \tag{4.17}$$

$$Hb(O_2)_2 + O_2 \overset{K^{(3)}}{\rightleftharpoons} Hb(O_2)_3 \qquad P_{1/2}^{(3)}(O_2) = 33[3.3]Torr \tag{4.20.4}$$

$$Hb(O_2)_3 + O_2 \overset{K^{(4)}}{\rightleftharpoons} Hb(O_2)_4 \qquad P_{1/2}^{(4)}(O_2) = 0.26[0.29]Torr \tag{4.20.5}$$

(0.6 mM hemoglobin A, bis(Tris) buffer, pH 7.4, 0.1 M Cl^-, 2 mM 2,3-DPG, 25 °C. The values in square brackets are affinities in Torr measured in the absence of 2,3- DPG.)

This simple scheme proposed by Adair[26] assumes that each of the four binding sites is identical. The $P_{1/2}(O_2)$ values given come from fitting the binding curve to this scheme.[27] When 2,3-DPG is removed, the affinity of hemoglobin for the first three molecules of Oz is substantially increased, and the degree of cooperativity is lowered (values in square parentheses). For progressively stronger binding, the following inequalities, reflecting the proper statistical weighting illustrated in Figure 4.5, should hold:

$$\frac{1}{4}K^{(1)} > \frac{4}{6}K^{(2)} > \frac{6}{4}K^{(3)} > \frac{4}{1}K^{(4)} \tag{4.18}$$

The $\frac{6}{4}$ ratio, for example, reflects the six equivalent forms of the doubly and the four equivalent forms of the triply ligated species. In other words, relative to a noncooperative system, at low O_2 availability dioxygen *release* is facilitated; at high O_2 availability dioxygen *binding* is facilitated. The scheme is readily extended to higher orders of oligomerization.

A simple model for analyzing cooperative ligand binding was proposed by Monod, Wyman, and Changeux in 1965, and is usually referred to as the MWC two-state concerted model.[28] Molecules are assumed to be in equilibrium between two conformations or quaternary structures, one that has a low ligand affinity and a second that has a high ligand affinity. The low-affinity conformation is often designated the T or tense state, and the high-affinity conformation the R or relaxed state. The equilibrium between the two conformations is characterized by the allosteric constant

$$L_0 = \frac{[R_0]}{[T_0]} \tag{4.19}$$

where the subscript denotes the unliganded Rand T states. The free-energy change upon binding a ligand to the R state, irrespective of saturation, is assumed to be a constant, and the associated equilibrium constant is designated K_R; a third constant, K_T, characterizes binding to the T state. Figure 4.7 illustrates this model, and introduces the terminology conventionally used.

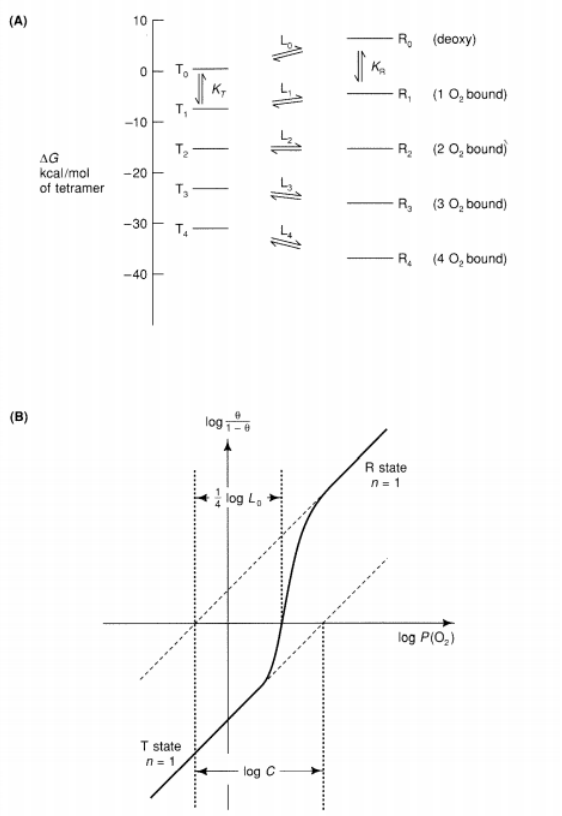

Figure 4.7 - The MWC two-state model for cooperative ligand binding:[4] (A) Free-energy relationships among R and T states; (B) Calculation of the allosteric constants from the binding curve.

To a reasonable approximation, the cooperative binding of dioxygen can be summarized by these three parameters, L_0, K_R, and K_T. The Adair constants may be expressed in terms of these parameters:

$$K^{(1)} = \frac{(1+L_0C)K_T}{1+L_0}, \qquad K^{(2)} = \frac{(1+L_0C^2)K_T}{1+L_0}, \tag{4.20}$$

$$K^{(3)} = \frac{(1+L_0C^3)K_T}{1+L_0C^2}, \qquad K^{(4)} = \frac{(1+L_0C^4)K_T}{1+L_0C^3}, \tag{4.20.6}$$

where $C = K_R/K_T$. The fractional saturation is given as

$$\theta = \frac{\alpha(1+\alpha)^3 + L_0\alpha C(1+\alpha C)^3}{(1+\alpha)^4 + L_0\alpha C(1+\alpha C)^4}, \tag{4.21}$$

where $a = K_T[X]$, and [X] is the concentration of the free ligand (e.g., O_2) in the same units (M or Torr) in which K_T is expressed. Figure 4.7B illustrates how the allosteric parameters, $C = K_R/K_T$ and $L_0 = [R_0]/[T_0]$, are extracted from a plot of saturation (as log [θ/(1 - θ)]) versus partial pressure of dioxygen (as log [$P(O_2)$]). Notice how the two-state model (Figure 4.7B) matches very closely the form of the binding curve for hemoglobin (Figure 4.4B). Equations (4.20) and (4.21) may be generalized to an oligomer with n subunits. In the case of hemoglobin, Perutz and coworkers,[11] through the determination of the crystal structures of a variety of hemoglobin derivatives, have given subsequently a sound structural basis to the MWC model of two basic quaternary states (see below).

A more exact treatment of ligand-binding data would allow for different affinities for different binding sites (called subunit heterogeneity) and different intrinsic affinities for ligand binding to the R-state conformation compared with the T-state conformation, for each level of ligand saturation—that is, for tertiary structure change within subunits upon ligation. This more exact treatment requires 25 separate equilibrium constants. Statistical thermodynamical approaches exist.[29] These explicitly incorporate the different types of subunit interactions that structural studies have revealed, and give improved fits to oxygen-binding data and to the Bohr effect. The key element of two basic quaternary states is preserved, at least for dioxygen binding.[29b]

For some modified hemoglobins, for example $[\alpha\text{-Fe(II)}_2[\beta\text{-Mn(III)}]_2$, where in the β subunits the heme iron is replaced by Mn(III), there is now strong evidence for three quaternary states,[29c] with the singly and several of the doubly ligated species having an energy state intermediate between the T (unliganded) and R (fully, triply, and the other doubly liganded) states.

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