The Physical Basis of Biochemistry: Reaction kinetics, enzyme catalysis and ligand binding equilibria

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1. Reaction kinetics: The quantitative description of the time course of chemical reactions

1.1. The definition of reaction velocity

After the start of any type of chemical reaction, the concentrations of the educts decrease and the concentrations of the products increase over time. For example, for a reaction type

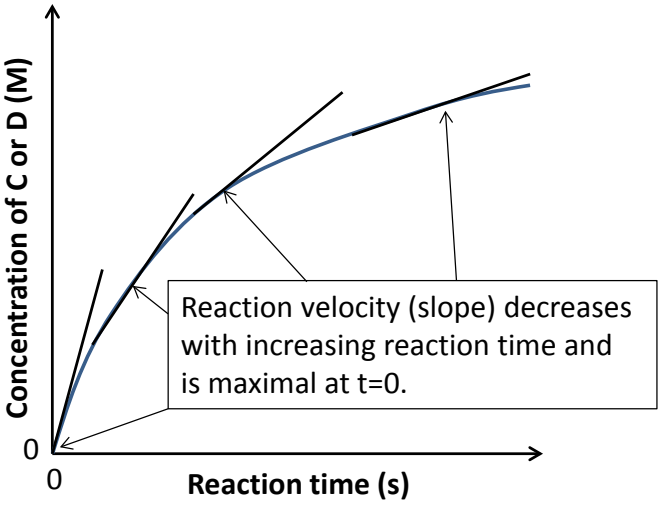
$$A + B \longrightarrow C + D$$

- the concentration of A decreases with the same rate as the concentration of B
- the concentration of C increases with the same rate as the concentration of D
- the concentration of A disappears with the same rate as the concentration of C or D increases
- the concentration of B disappears with the same rate as the concentration of C or D increases

This is due to the mechanism of the reaction, according to which C or D can only be formed when A and B react with each other. To indicate the velocity v of a chemical reaction, it is therefore sufficient to indicate only the chance in the concentration over time of one of the components of the reaction (A, B, C, or D in this example):

$$v = -\frac{d[A]}{dt} = -\frac{d[B]}{dt} = \frac{d[C]}{dt} = \frac{d[D]}{dt}$$

The reaction velocity v is thus the change in the concentration of a reactant over time and has the unit M s^{-1} or mol L^{-1} s^{-1}. The following diagram shows the formation of the reaction product C over time as an example. It demonstrates that the reaction velocity decreases with reaction time. The reaction velocity is highest at the beginning of the reaction (reaction time zero). It is then termed **i**nitial **v**elocity (v_i).



1.2. First Order Reactions

The simplest case of a first order reaction is the irreversible reaction:

$$A \xrightarrow{k} B$$

Examples: Radioactive decay, the spontaneous unfolding of a protein from the native (N) state directly to the unfolded state (U), or the spontaneous dissociation of a protein/ligand complex into free protein and ligand ($PL \rightarrow P + L$).

The velocity v of the reaction at time point t is proportional to the concentration of A that exists at time point t.

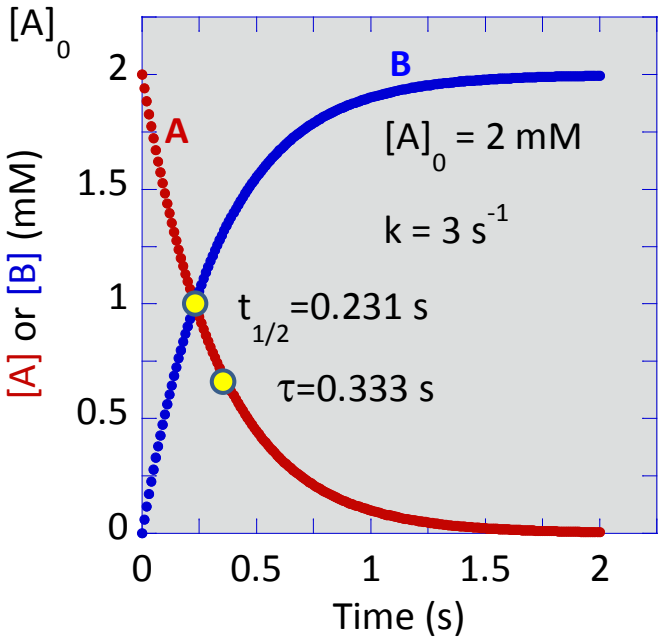
$$v = -\frac{d[A]}{dt} = k \cdot [A]$$

After integration with the limits [A] = A_0 (starting concentration at time zero) and [A] the concentration of A at any time point t is:

$$\int_{[A]=A_0}^{[A]} \frac{d[A]}{[A]} = k \cdot \int_0^t dt \qquad -\ln\frac{[A]}{A_0} = k \cdot t \qquad \boxed{[A] = A_0 \cdot e^{-k \cdot t}}$$

The unit of the first-order rate constant is (s^{-1}).

Example: $A \rightarrow B$; $k = 3\ s^{-1}$



For any irreversible first-order reaction $A \rightarrow B$, the time intervals that are necessary for going to half the concentration of A are identical throughout the curve. This time is called the half-life ($t_{1/2}$) of the reaction. Replacing t by $t_{1/2}$ and [A] by $[A_0]/2$ yields

$$t_{1/2} = \frac{\ln 2}{k} = \frac{0.693}{k} = 0.693 \cdot \tau$$

The „time constant" τ (unit: s) is the reciprocal value of the first-order rate constant k and often used instead of k for describing first-order reactions, as τ roughly equals the half-life.

Note that the half-life of first-order reactions $A \rightarrow B$ is independent of the concentration of A at any time of the reaction and only dependent on k.

The initial velocity v_i of a first-order reaction is given by the equation

$$v_i = k \cdot [A_0]$$

Thus, the initial velocity of a first-order reaction increases linearly with $[A_0]$ the initial concentration of A, but its half-life is independent of $[A_0]$.

1.3. Second-Order Reactions

A second order reaction occurs when the productive collision of two reaction partners A and B is required for the reaction to occur. The rate at any time t is dependent on the concentration of both reaction partners at time t and the proportionality constant k (second-order rate constant) that has the unit $M^{-1}s^{-1}$.

$$A + B \xrightarrow{k} C$$

$$\frac{d[A]}{dt} = -k \cdot [A] \cdot [B]$$

The simplest case for such a reaction occurs when the initial concentrations of A and B are identical ($[A_0]=[B_0]$). Then the above equation simplifies to:

$$-\frac{d[A]}{dt} = k \cdot [A]^2$$

To evaluate experimental data, the integrated form is used:

$$-\int_{[A]=A_0}^{[A]} \frac{d[A]}{[A]^2} = k \cdot \int_0^t dt$$

$$\frac{1}{[A]} - \frac{1}{[A_0]} = k \cdot t \quad \Leftrightarrow \quad [A] = \frac{1}{kt + \frac{1}{[A_0]}}$$

Replacing t by $t_{1/2}$ and [A] by $[A_0]/2$ yields

$$t_{1/2} = \frac{1}{k[A_0]}; \quad (A+B \rightarrow C;\ [A_0]=[B_0])$$

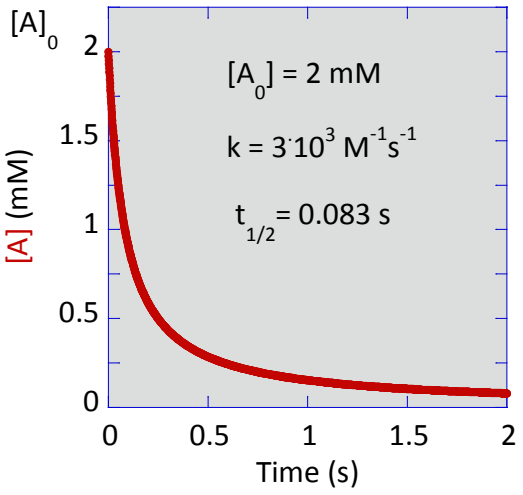
This equation shows that the half-life of second-order reactions *is dependent on concentration*. If $[A_0]=[B_0]$, the first half-life is $1/(k[A_0])$. The "initial concentration" for the rest of the reaction is then only $[A_0]/2$, which means that the next half-life will be two times longer than the first half life. Thus, there is a doubling of the half-life after each half-life.

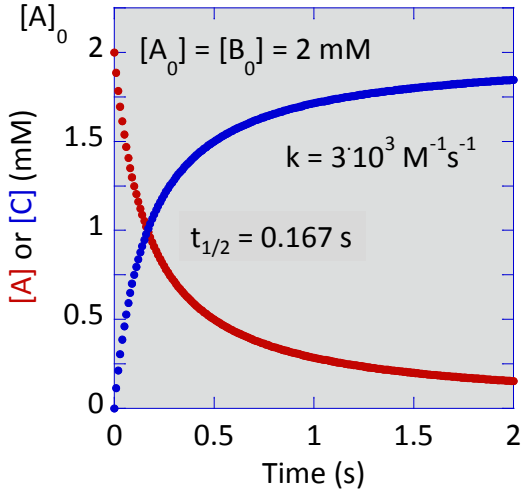
Note that, in contrast to a reaction between two different molecules (A + B → C), the half-life of a homodimerization reaction (A + A → C) is two-fold smaller, because every molecule A will always collide with another molecule A.

$$t_{1/2} = \frac{1}{2k[A_0]}; \quad (A + A \rightarrow C)$$

The following diagrams illustrate the difference between a homo-dimerization reaction and the formation of a heterodimer (with $[A_0] = [B_0]$) for the case that both reactions have the same second-order rate constant of $3 \cdot 10^3$ $M^{-1}s^{-1}$ and start with initial concentrations of 2 mM:

$$A + A \xrightarrow{k} C \qquad A + B \xrightarrow{k} C$$





The initial velocity v_i of a second-order reaction A + B $\rightarrow$ C is given by the equation

$$v_i = k \cdot [A_0] \cdot [B_0]$$

Doubling the initial concentrations of A and B will thus increase the initial velocity fourfold, and decrease the first half-life two-fold.

Pseudo-First Order Reactions are second-order reactions in which one of the two components is present at vast excess over the other.

Often it is not possible to choose identical starting concentrations for the two reaction partners A and B. The most frequently applied practical solution to this problem is to set the initial conditions such that one of the reaction partners is present at large excess over the other (e.g. $[A_0] >> [B_0]$). In this case, the concentration of A can be considered constant during the course of the reaction, i.e., $[A] \approx [A_0]$. The product of k and $[A_0]$ can then be combined to an apparent pseudo first-order rate constant k_{pseudo}:

A + B $\rightarrow$ C; $[A_0] >> [B_0]$:

$$k_{pseudo} = k[A_0] \text{ ; unit: } s^{-1}$$

$$-\frac{d[B]}{dt} = k \cdot [A_0] \cdot [B] \quad \Leftrightarrow \quad -\frac{d[B]}{dt} = k_{pseudo} \cdot [B]$$

Integration yields:

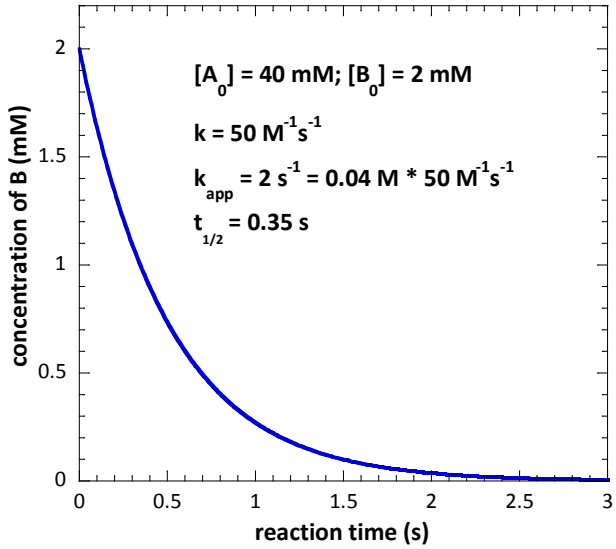
$$[B] = [B_0] \cdot e^{-k_{pseudo} \cdot t}$$

In practice, pseudo-first order reactions thus look like first-order reactions and show a constant half-life over the entire course of the reaction.

$$t_{1/2} = \frac{\ln 2}{k_{pseudo}} = \frac{\ln 2}{k \cdot [A_0]}$$

Thus, the half-life of pseudo-first order reactions is only dependent on the second-order rate constant k and the concentration of the compound that is present at excess over the other.

An example with 20-fold excess of A over B at the beginning of the reaction is shown in the following diagram.



The upper limit of second-order reactions in water

It is obvious that a second order reaction in aqueous solution cannot occur faster than the average time required for stochastic collision between A and B. If any collision between A and B was productive and led to product formation, the reaction velocity would be "diffusion controlled". For molecules (except H^+ and OH^-) in water, the corresponding upper limit for the second-order rate constant is about $\mathbf{7 \cdot 10^9\ M^{-1}s^{-1}}$ for small molecules and about $\mathbf{10^8–10^9\ M^{-1}s^{-1}}$ for protein-ligand interactions. It follows that diffusion-controlled reactions are extremely fast. As an example, we calculate the half-life of a diffusion controlled second-order reaction (A + B→ C) for the case that the reaction is started with identical initial concentrations of $[A_0] = [B_0] = 1\ \mu M$. The half-life is given by $t_{1/2} = 1/(k \cdot [A_0])$. With $k = 10^9\ M^{-1}s^{-1}$ and $[A_0] = 10^{-6}$ M, a half-life of 1 ms is obtained. Surprisingly, the very dense intracellular environment (high concentrations of certain metabolites and total protein concentrations of up to 300 mg/ml) does not slow intracellular diffusion dramatically. For example, a protein of average size only needs about 10 ms to traverse an *E. coli* cell and only about 10 s to traverse a human HeLa cell. Note that the time required for a molecule to diffuse over a certain distance increases with the *square* of the distance!

Second-order rate constants of physiologically relevant reactions are typically in the range of $10^4–10^7\ M^{-1}s^{-1}$. In turn, smaller values of second-order rate constants indicate that the corresponding reaction is not physiological.

For unimolecular (intramolecular) first-order reactions, there is also a theoretical upper limit. It is assumed that the rate constant of unimolecular reactions cannot be higher than the frequency of a molecular vibration, which is in the order of $10^{12}–10^{13}\ s^{-1}$.

1.4. Zero-order reactions

Remember that the *reaction velocity* v at any given time point is concentration dependent for both unimolecular and biomolecular reactions:

unimolecular: v = k[A]: bimolecular: v = k[A][B]

The reaction velocity of a zero-order reaction is, in contrast, entirely independent of reactant concentration:

$$v = -\frac{d[A]}{dt} = k$$

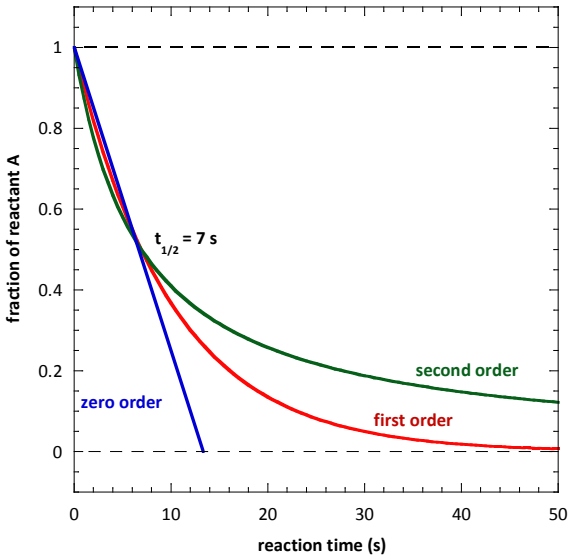
where k has the unit of the reaction velocity, namely M s^{-1}.

Zero-order reactions are generally only observed for catalyzed reactions when

I. the spontaneous reaction is so slow that it cannot be observed,
II. the reaction only occurs when the reactant is bound to the surface of a catalyst and
III. all binding sites of the catalyst are fully occupied with reactant throughout the reaction (full saturation of the catalyst with substrate).

These conditions apply to reactions catalyzed by enzymes when the substrate concentrations are more than 100-fold higher than the K_M value. A practical example is the degradation of alcohol in the blood by alcohol dehydrogenase. The alcohol degradation rate is approximately 0.1‰ per hour and independent of alcohol concentration. It is therefore impossible to increase the velocity of alcohol degradation by drinking more alcohol.

The following figure shows that the order of a chemical reaction is often evident from the shape of the reaction profile. It shows the comparison of a zero-order, first-order and second-order reaction (with $[A_0]=[B_0]$) that all have the same first half life of 7 s. The second-order reaction is readily recognized by the doubling of the half-life after the first half-life (i.e., the decrease of A from $[A_0]$ to 0.5 $[A_0]$ takes 7 s, and from 0.5 $[A_0]$ to 0.25 $[A_0]$ takes 14 s). In other words, second-order reactions proceed very slowly towards the end of the reaction.



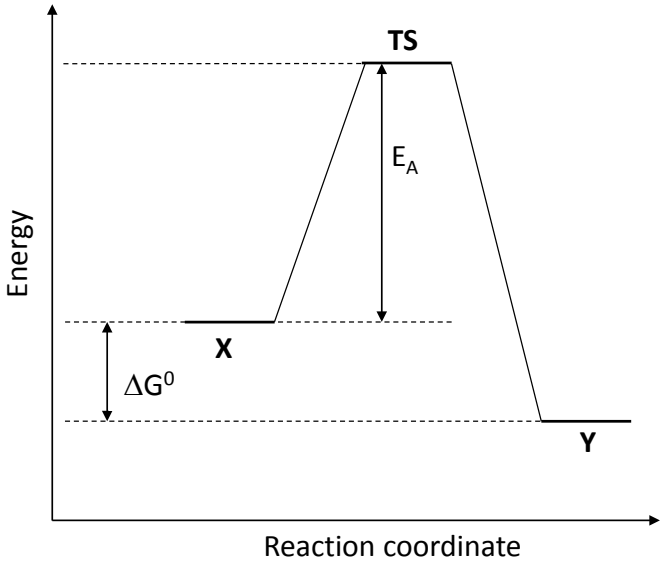
2. Enzyme Kinetics

2.1. The activation energy of chemical reactions and transition state stabilization by enzymes

Any chemical reaction proceeds via one or several transition states. Let us consider the simplest case, i.e., a unimolecular equilibrium between two states X and Y (X ↔ Y) with a single transition state (TS). The energy difference between X and Y is given by the Gibbs equation:

$$\Delta G^0 = -RT \ln ([Y]/ [X])$$

The energy diagram of this reaction can be described as follows:



The transition state is defined as a state of higher energy relative to educt (X) and product (Y) that, although it cannot be detected directly, needs to be populated transiently for the reaction to occur. In the case of a reversible equilibrium X ↔ Y, both the forward and the reverse reaction have the same transition state. Let us assume that X is much more stable than Y so that we can neglect the reverse reaction. The reaction then simplifies to an apparently irreversible reaction

$$X \xrightarrow{k} Y$$

For the reaction to occur, any molecule first needs to reach TS and overcome the activation energy of the reaction (E_A), which is the energy difference between X and TS. According to the Boltzmann distribution (Gibbs equation), the ratio between molecules in the states TS and X under equilibrium conditions is given by

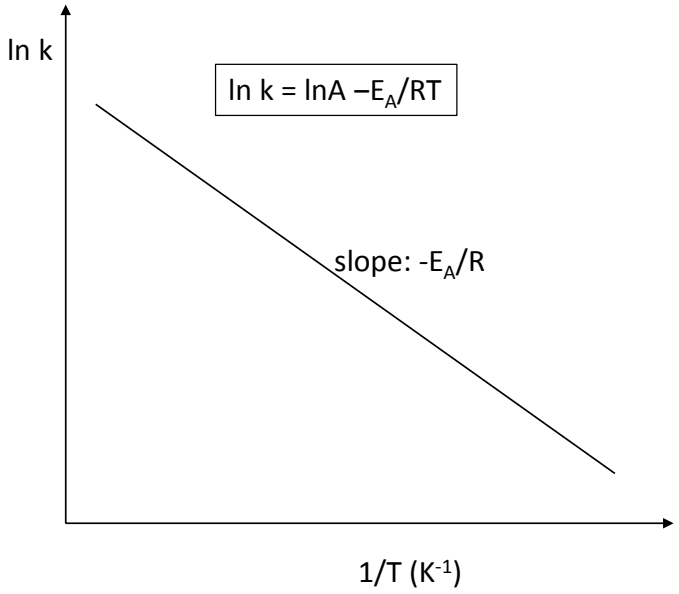
$$\frac{[\mathrm{TS}]}{[\mathrm{X}]} = \mathrm{e}^{-E_A/RT}$$

As the reaction velocity v equals $[X]\cdot k$ and is directly proportional to [TS], we can write

$$k \sim \mathrm{e}^{-E_A/RT} \quad \text{or}$$

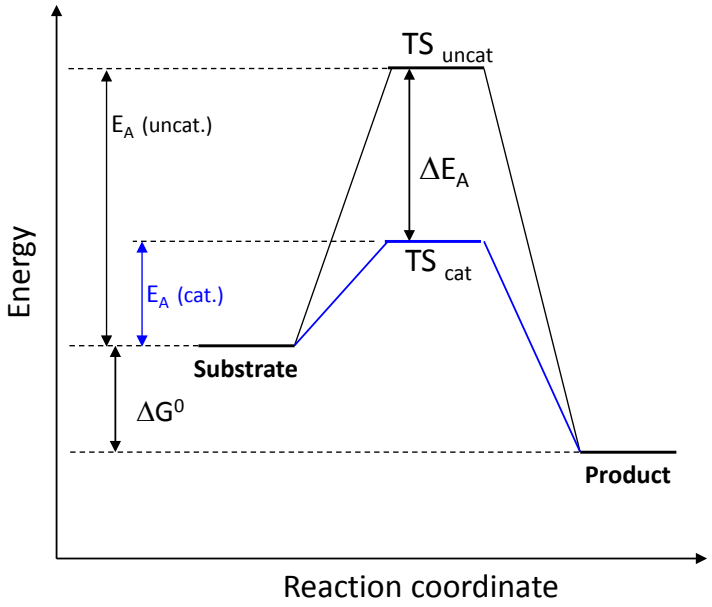
$$k = \mathrm{A} \cdot \mathrm{e}^{-E_A/RT} \textit{ (Arrhenius equation)}$$

The pre-exponential factor A defines the theoretical upper limit of the rate constant (rate constant at zero E_A), and the term $\mathrm{e}^{-E_A/RT}$ is factor by which the observed rate constant at temperature T is lowered by E_A. The logarithmic form of the above equation yields a linear decrease of ln k with increasing values of the inverse temperature, showing that the *activation energy of a chemical reaction can determined experimentally via the temperature dependence of its rate constant*:



It follows that reactions with high E_A show high temperature dependence, and reactions with low E_A have low temperature dependence.

Many reactions in metabolism, although energetically favorable, show very high activation energy barriers. In the absence of enzymes, their reaction half-lives at physiological temperature can be in the range of millions of years. The acceleration of essentially all metabolic reactions in the cell by specific enzymes is therefore indispensable for life and cellular metabolism. As a matter of fact, many enzymes exhibit spectacular catalytic power and can accelerate biochemical reactions up to 10^{17}-fold relative to the uncatalyzed (spontaneous) reaction. The rate enhancement is accomplished by lowering the activation energy (E_A) of the reaction via specific stabilization of the transition state (TS) of the reaction:



Energy diagram for an uncatalyzed and a catalyzed reaction. The enzyme lowers the activation energy by ΔE_A, while not affecting the equilibrium and free energy difference (ΔG) between substrate and product.

The factor by which the enzyme accelerates a reaction equals the ratio between the rate constant with which an enzyme-bound substrate reacts to the product (k_{cat}, see also below) and the rate constant of the uncatalyzed reaction (k_{uncat}). The acceleration factor (k_{cat}/k_{uncat})is linked to the energy by which the enzyme lowers the activation energy of the reaction, ΔE_A, by the following equation:

$$\frac{k_{cat}}{k_{uncat}} = e^{\frac{\Delta E_A}{R \cdot T}}$$

This relationship is analogous to the Gibbs equation, where an equilibrium constant of 10:1 for a two-state equilibrium translates into a free energy difference of 5.7 kJ mol^{-1} between the two states. In other words, the catalyzed reaction is 10-fold faster than the uncatalyzed reaction when the enzyme lowers the activation energy by 5.7 kJ mol^{-1}. Thus, the acceleration factor of 10^{17} measured for the enzyme orotidine 5'-phosphate decarboxylase (Radzicka and Wolfenden, Science 267, 90–93, 1995) corresponds to a reduction of the activation energy through the enzyme by 17 x 5.7 kJ mol^{-1} = 96.9 kJ mol^{-1}.

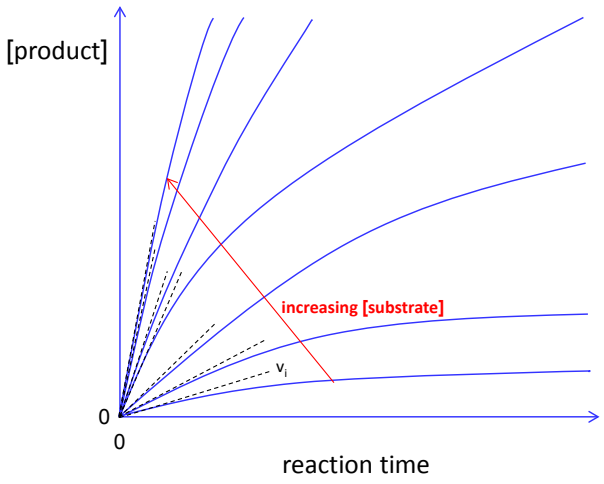
2.2 Definition of a catalyst

Enzymes are catalysts that accelerate metabolic reactions. Like any other catalyst in chemistry, they meet the following criteria:

- Enzymes accelerate chemical reactions by lowering the activation energy.
- Enzymes leave the reaction they are catalysing in the same state in which they entered the reaction.
- Enzymes act in ***sub***stoichiometric (catalytic) amounts because an individual enzyme catalyses the same reaction over and over again (multiple turnovers).
- Under conditions where the substrate concentration is lower than enzyme concentration ($[S_0]>>[E_0]$), the velocity of the catalyzed reaction increases linearly with enzyme concentration.
- Enzymes show saturation behaviour at high substrate concentrations: When all binding sites are occupied with substrate, the maximum reaction velocity (v_{max}) is reached and stays constant even when substrate concentration is further increased (zero order reaction conditions, see 1.4.).
- Enzymes accelerate the forward and reverse reaction by the same factor. *Therefore, they do not change the equilibrium (energy difference) between substrate and product, but they accelerate the attainment of the equilibrium between substrate and product.*

2.3 Michaelis Menten Kinetics and Experimental Observations in Enzyme Reactions

Almost all metabolic reactions are carried out with the help of enzymes. The poperties of an enzyme can be characterized *in vitro* with a series of experiments in with a constant amount of enzyme is mixed with different amounts of substrate (constant enzyme concentration, various substrate concentrations) and the kinetics of product formation are recorded. If the uncatalyzed reaction S→P is so slow that it cannot be observed experimentally, a typical data set will look as follows:



The result shows that the *initial velocity* v_i of product formation first increases with increasing substrate concentrations and then reaches a maximum at very high substrate concentrations.

This "saturation" is reached when all enzyme molecules are completely saturated with substrate. A further increase of substrate concentration will then have no effect on the observed initial velocity, and zero order reaction conditions (see 1.4.) will be reached.

Initial velocities are typically recorded within the first 10% of the reaction where one can observe a linear increase in product concentration with time. This linearity is due to the fact that the occupancy of the enzyme with substrate does not change significantly in the time window in which v_i is measured (steady state conditions, substrate concentration decreases by less than 10% during measurement if v_i so that $[S] \approx [S_0]$).

2.4. k_{cat}, K_M and k_{cat}/K_M: The meaning of the Michealis Menten Equation

We now consider the simplest case of a reaction catalyzed by an enzyme. We assume that the uncatalyzed reaction is not observed and that the product P is thermodynamically much more stable than the substrate S. In this case, the reverse reaction (P→S) becomes negligible, and the following reaction scheme is obtained:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P$$

The Michaelis Menten equation describes the dependence of the recorded initial velocity on substrate concentration and enzyme concentration:

$$v_i = v_{max} \frac{[S]}{K_M + [S]}, \text{ where } v_{max} = k_{cat} \cdot [E_0]$$

E_o= total enzyme concentration

The Michaelis constant K_M has the unit of a concentration (M) and is defined as follows:

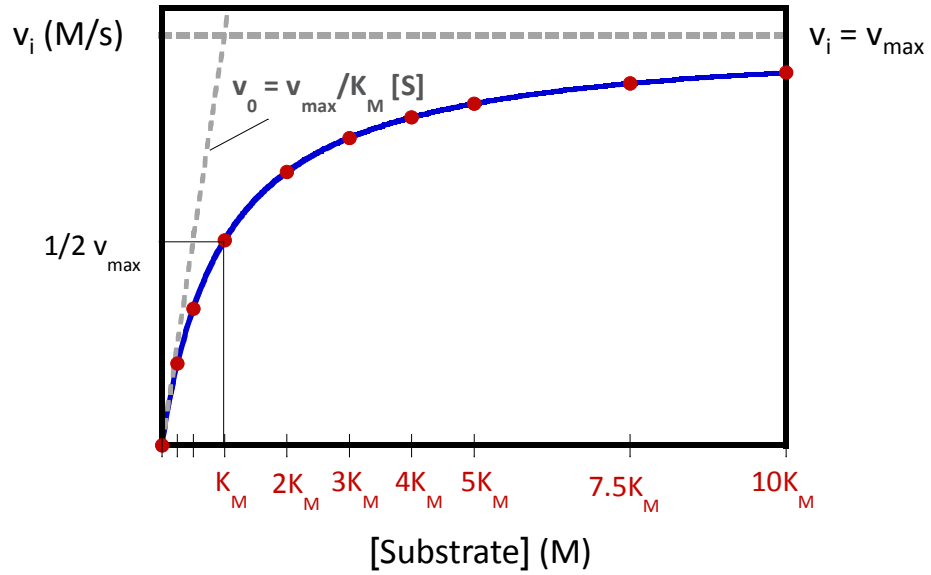
$$K_M = \frac{k_{-1} + k_{cat}}{k_1}$$

The maximum reaction velocity v_{max} refers to the situation where the enzyme is fully occupied with substrate ($[ES]=[E_0]$).

The term $[S]/(K_M+[S])$ is always a number between zero and 1 and corresponds to the factor by which v_{max} is diminished at incomplete saturation of the enzyme with substrate, as illustrated by the following table:

[substrate]	v_i	saturation of E with S
0.01 K_M	0.01 v_{max}	1%
0.1 K_M	0.09 v_{max}	9%
K_M	0.5 v_{max}	50%
10 K_M	0.91 v_{max}	91%
100 K_M	0.99 v_{max}	99%

A plot of initial velocity against against substrate concentration is hyperbolic and illustrates enzyme saturation at high substrate concentrations:



Thus, the Michaelis constant K_M indicates the substrate concentration at which the enzyme works with $v_{max}/2$. The K_M is thus an apparent dissociation constant (see below) that may be treated as the overall dissociation constant for all enzyme-bound species. For enzymes/substrate pairs where k_{cat} is much smaller than k_{-1}, the equation simplifies to $K_M = k_1/k_1$ and becomes formally identical to a dissociation constant of a noncovalent protein-ligand complex. Thus, the K_M value is related to the affinity of an enzyme for its substrate and decreases with increasing affinity. K_M values of natural enzyme-substrate pairs are typically in the range of 10^{-3}–10^{-6} M.

The turnover number k_{cat} (unit: s^{-1}) indicates the number of substrate molecules converted into product per enzyme molecule and second at full saturation with substrate. The k_{cat} is a first-order rate constant that refers to the spontaneous dissociation of ES to E and P. The k_{cat} cannot be any greater than any first-order rate constant on the forward reaction pathway. It sets a lower limit for all unimolecular reactions in the catalytic cycle of the enzyme. In other words, k_{cat} is the slowest unimolecular step in the catalytic cycle of an enzyme. The highest values of k_{cat} that have been found so far in enzymes are in the range of 10^5-10^6 s^{-1}. The reciprocal of k_{cat} is the time required to convert a single substrate molecule to product in the steady state at saturating [S].

The specificity constant k_{cat}/K_M is an apparent second-order rate constant (unit: $M^{-1}s^{-1}$) that refers to the properties and the reactions of the free enzyme and free substrate. It is the classical parameter used for quantifying the catalytic efficiency of an enzyme. The value of k_{cat}/K_M cannot be greater than that of any second-order rate constant on the forward reaction pathway. It thus sets a lower limit on the rate constant for the association of enzyme and substrate. Analogous to second-order reactions in aqueous solution, the maximum theoretical value of k_{cat}/K_M is ~ 10^9 $M^{-1}s^{-1}$, i.e. the value of a diffusion controlled second-order reaction.

Highly efficient enzymes (e.g. acetylcholinesterase, carbonic anhydrase, catalase, triosephosphate isomerase, β-lactamase) have k_{cat}/K_M values of ~10^8 $M^{-1}s^{-1}$. The most efficient enzyme known is superoxide dismutase, with $k_{cat}/K_M = 7 \cdot 10^9$ $M^{-1}s^{-1}$.

Another parameter for characterizing an enzyme's performance is $k_{cat}/(K_M \cdot k_{uncat})$ (unit: M^{-1}), which takes the acceleration of the reaction by the enzyme relative to the uncatalyzed reaction into account. As a rough approximation, one can consider $k_{cat}/(K_M \cdot k_{uncat})$ equivalent to the affinity constant ($1/K_{Diss}$) of the enzyme for the transition state of the reaction.

One reason why the parameter $k_{cat}/(K_M \cdot k_{uncat})$ has been introduced is the fact that the known rates of uncatalyzed metabolic reactions under physiological conditions differ by up to 14 orders of magnitude. For example, the spontaneous decarboxylation of orotic acid occurs with a half-life of almost 100 million years, while e.g. the half-life of the spontaneous trans-to-cis isomerization of a prolyl peptide bond is only about 100 s. This means that there are enormous differences between natural enzymes with respect to their ability of lowering the activation energy barrier for the reaction they are catalyzing, and enzymes with very high k_{cat}/k_M values are not necessarily the best enzymes in terms of $k_{cat}/(K_M \cdot k_{uncat})$. OMP carboxylase has the highest $k_{cat}/(K_M \cdot k_{uncat})$ value reported for an enzyme so far (10^{23} M^{-1}).

An additional parameter for quantifying the performance of an enzyme is k_{cat}/k_{uncat}, i.e., the factor by which it accelerates the catalyzed reaction at full saturation with substrate relative to the uncatalyzed reaction (k_{uncat}). As mentioned above, the enzyme orotidin-5'phosphate decarboxylase (OMP decarbocylase), with a k_{cat}/k_{uncat} value of 10^{17}, is the most proficient enzyme in this respect.

How are Michaelis Menten kinetics described if an enzyme has two substrates and two products? Let us consider the enzyme hexokinase which catalyzes the conversion of ATP and glucose to ADP and glucose-6-phosphate:

$$\text{Glucose} + \text{ATP} \xrightarrow{[\text{Hexokinase}]} \text{Glucose-6-phosphate} + \text{ADP}$$

Hexokinase has a specific binding site for glucose and a specific binding site for ATP. The glucose concentration determines the saturation of the glucose binding site and the ATP concentration determines the saturation of the ATP binding site. V_{max} can therefore only be attained if both binding sites are fully saturated. Consequently, the initial velocity of the catalyzed reaction depends on the concentrations of hexokinase, glucose and ATP, the K_M values for glucose and ATP, and k_{cat}:

$$v_i = k_{cat} \cdot [E_0] \cdot \left[\frac{[\text{Glucose}]}{K_{M\,\text{glucose}} + [\text{Glucose}]} \right] \cdot \left[\frac{[\text{ATP}]}{K_{M\,\text{ATP}} + [\text{ATP}]} \right]$$

The equation shows that the enzyme only works with 0.25 v_{max} if [Glucose] = $K_{M\ glucose}$ and [ATP] = $K_{M\ ATP}$. If both binding sites are 50% occupied, only 25% of the enzyme molecules will be occupied with both substrates, which is a requirement for catalysis. In order to determine the enzymatic parameters of hexokinase correctly, it is therefore necessary to determine the K_M values of both substrates independently: In a first set of experiments, the dependence of v_i on [Glucose] is measured at constant [ATP], in a second set of experiments, the dependence of v_i on [ATP] is measured at constant [glucose]. V_{max} can then be verified under conditions near full saturation with both substrates, and k_{cat} can be calculated from $k_{cat} = v_{max}/[E_0]$.

3. Protein-ligand binding equilibria

The function of proteins relies on binding of interaction partners: small molecule ligands (nucleotides, metabolites, metal ions etc.), other proteins (receptors, regulators, heterooligomeric complexes etc.), DNA (repressors) and RNA. Purpose of this initial interaction can be: catalysis of a reaction of the ligand (chemical transformation), activation/inactivation of ligand (signalling), transport or storage of the ligand or formation of higher order structures.

The ligands bind non-covalently to the protein. Consequently, an equilibrium between bound and free species is established.

Binding is based on steric and chemical complementarity between protein and ligand. Binding sites on proteins generally are on the surface or close to it. Interactions with other proteins often occur through flat faces on the proteins, interactions with very large molecules like DNA often occur via protruberances. The ligand forms H-bonds, electrostatic interactions, van der Waals interactions and hydrophobic interactions with the protein residues in the binding site.

Frequently, binding of the ligand induces a conformational change in the protein.

Two rate constants define a simple binding equilibrium: The second-order rate constant k_{on} for the association of the protein P with its ligand L to the protein-ligand complex PL, and the first-order rate constant k_{off} describing the rate of spontaneous dissociation of PL:

$$P + L \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} PL$$

After mixing of P and L, the binding equilibrium is attained when the concentrations of P, L and PL do not change anymore. This is the case when PL forms and dissociates with the same velocity:

$$k_{on} \cdot P \cdot L = k_{off} \cdot PL$$

where P and L are the concentrations of protein and ligand *after attainment of the equilibrium*, respectively.

The ratio k_{off}/k_{on} equals the *dissociation constant* of the PL complex, K_{Diss}, which has the unit of concentration (M). Alternatively, the *association constant* K_a can be used to describe the strength of interaction of a protein-ligand complex. K_a is defined as K_{Diss}^{-1} and has the unit M^{-1}.

Dissociation constant: $$K_{Diss} = \frac{k_{off}}{k_{on}} = \frac{P \cdot L}{PL} = \frac{1}{K_a} \quad \text{(unit: M)}$$

For a quantitative description of the occupancy of a protein with ligand as a function of the total protein concentration P_{tot}, the total ligand concentration L_{tot} and K_{Diss}, we consider the following cases:

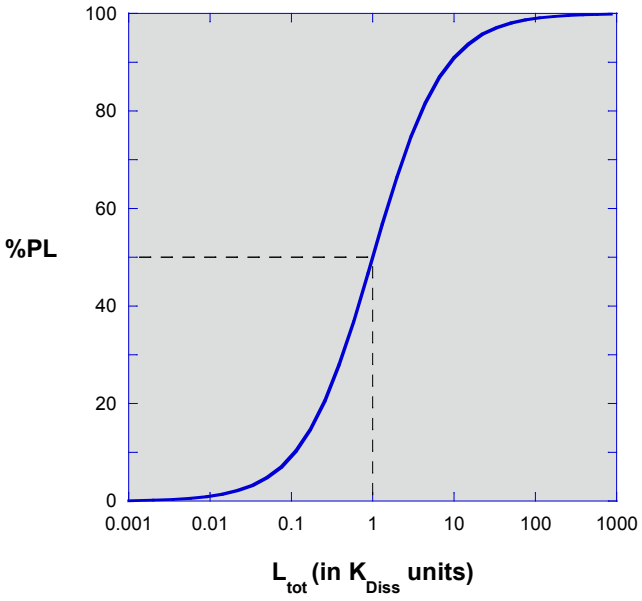
Case 1: The total ligand concentration is much higher than the total protein concentration

We assume that we have a solution with P and L where the total concentration of P (P_{tot}) is much smaller than L_{tot}. This means the concentration of free ligand, L, will not decrease significantly even if all P molecules were occupied with L. We thus can make the approximation that the concentration of free ligand (L) after attainment of equilibrium is equal to L_{tot}, and we can write

$$K_{Diss} = \frac{P \cdot L_{tot}}{PL} \quad \text{or} \quad \frac{K_{Diss}}{L_{tot}} = \frac{P}{PL}$$

Consequently, under the condition $L_{tot} >> P_{tot}$, K_{Diss} indicates the ligand concentration required to achieve 50% saturation of P with L. The lower K_{Diss}, the higher is the affinity of the protein-ligand complex (i.e., the less ligand is required to reach 50% occupancy). The above equation allows the calculation of the occupancy of the protein with ligand at any ligand concentration:

L_{tot}	P/PL	%P occupied with L
100 K_{Diss}	1:100	99%
10 K_{Diss}	1:10	91%
K_{Diss}	1:1	50%
K_{Diss} /10	10:1	9%
K_{Diss} /100	100:1	1%



The K_{Diss} values of natural protein-ligand complexes vary over a wide range, typically between 10^{-3} M (enzyme/substrate interactions) and 10^{-15} M (avidin/biotin complex), but can even reach values of 10^{-20} M in the case of extracellular protein-protein complexes (e.g. filamentous pili of bacteria). Differences in K_{Diss} between natural protein-ligand complexes mainly result from differences in k_{off}, while the values of k_{on} are often similar, in the range of 10^4–10^7 $M^{-1}s^{-1}$. As an example, the half-life of spontaneous dissociation of the avidin/biotin complex (K_{Diss} = 10^{-15} M) is about one week.

Case 2: Similar total concentrations of protein and ligand

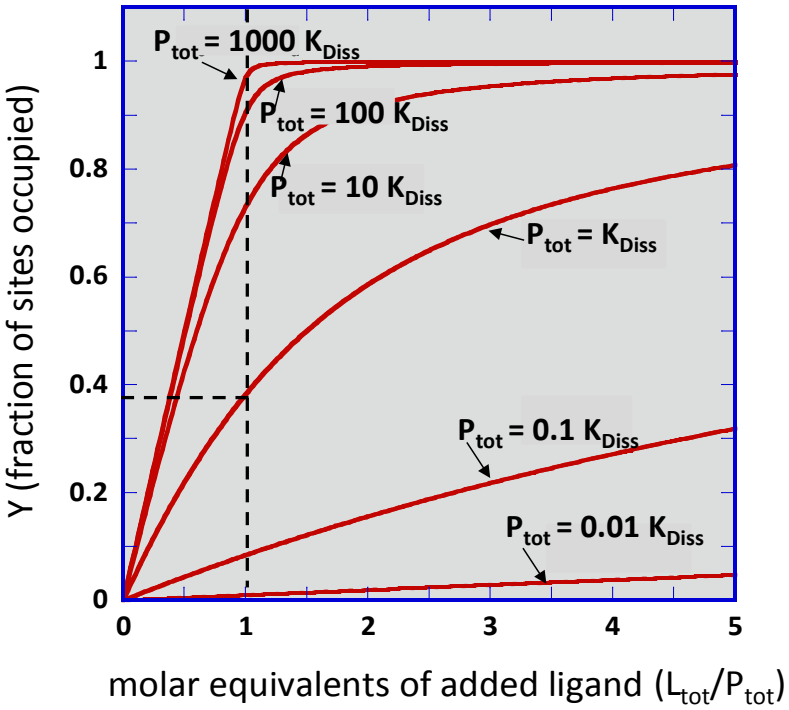
The quantitative description of a noncovalent protein/ligand binding equilibrium becomes more complex when the assumption $[P_{tot}] << [L_{tot}]$ does no longer hold, i.e., when $[P_{tot}]$ is similar to $[L_{tot}]$. Under these conditions, the fraction of protein occupied with ligand (y), not only depends on K_{Diss} and $[L_{tot}]$, but also on $[P_{tot}]$:

$$K_{Diss} = \frac{P \cdot L}{PL}; \quad P_{tot} = P + PL; \quad L_{tot} = L + PL;$$

Combination of these equations yields to the following equation for calculating y:

$$y = \frac{PL}{P_{tot}} = \frac{(K_{Diss} + L_{tot} + P_{tot}) - \sqrt{(K_{Diss} + L_{tot} + P_{tot})^2 - 4 \cdot P_{tot} \cdot L_{tot}}}{2 \cdot P_0}$$

This equation is generally valid and can be applied to any protein/ligand binding equilibrium. It simplifies to $K_{Diss} = P \cdot L_{tot}/PL$ if $L_{tot} >> P_{tot}$. The following figure shows an equilibrium titration experiment in which the protein concentration is kept constant, increasing amounts of ligand are added and the occupancy of the protein with ligand, y, is measured. The figure shows the result of such a titration experiment for different ratios between P_{tot} (which is kept constant in each titration curve) and K_{Diss}.



The diagram shows that the statement "K_{Diss} is the ligand concentration required to reach 50% occupancy with ligand" is no longer valid if $P_{tot} \approx L_{tot}$. Specifically, at similar total concentrations of protein and ligand, *y depends on the P_{tot}/K_{Diss} ratio*. For example, if $P_{tot} = L_{tot} = K_{Diss}$, only 38% of the protein molecules will be occupied with ligand, and if $P_{tot} = L_{tot} = 10\ K_{Diss}$, ~75% of the protein molecules will be occupied with ligand. As the information on the accurate K_{Diss} values

is contained in the *curvature* of the experimentally determined titration profile, it is also evident that it is no longer possible to determine K_{Diss} values accurately when P_{tot} is larger than 100 K_{Diss}. In practice, the minimum protein concentration required to measure its occupancy with ligand in solution with spectroscopic or other techniques is about 10^{-8} M. It follows that it is notoriously difficult to determine correct K_{Diss} values of high-affinity protein ligand complexes by equilibrium titration experiments. In the case of the biotin/avidin complex, for example, the minimum protein concentration of 10^{-8} M to detect ligand binding would still be 10^{7}-fold above the K_{Diss} (10^{-15} M). A titration experiment would simply result in a straight line with a kink at a biotin/avidin ratio of 1:1 (i.e. 100% occupancy at a 1:1 ratio; no curvature). Therefore, alternative methods are needed to get accurate K_{Diss} values of high-affinity complexes. As $K_{Diss} = k_{off}/k_{on}$, the problem can e.g. be solved by measuring k_{on} and k_{off} independently.

Conclusions:

- For the experimental determination of K_{Diss} values, the situation $P_{tot} << L_{tot}$ mainly applies to low-affinity protein/ligand complexes, where very high concentrations of L (high excess of L over P) are required to achieve saturation of protein with ligand. If $P_{tot} << L_{tot}$, y only depends on L_{tot} and K_{Diss}.

- For determining K_{Diss} values of high-affinity protein-ligand complexes, where a small excess of L over P may already be sufficient to saturate P with L, the general equation described for case 2 applies. Here, y notonly depends L_{tot} and K_{Diss}, but also P_{tot}!

4. Realization of energetically unfavorable metabolic reactions

Many steps in biosynthetic pathways are energetically unfavorable. As enzymes only accelerate the attainment of chemical equilibria but do not change the equilibria, catalysis of an energetically unfavorable reaction by an enzyme is not sufficient to drive this reaction. There are two main mechanisms that allow energetically unfavorable reactions to occur:

4.1. Coupling of an energetically favorable reaction to an unfavorable reaction.

We consider the first step in glycolysis, the conversion of glucose and phosphate to glucose-6-phosphate. This reaction is energetically unfavorable with a positive ΔG^0 value of +13.8 kJ/mol. In the cell, the formation of glucose-6-phosphate is coupled to the hydrolysis of ATP to ADP and phosphate, which is energetically highly favorable with a negative ΔG^0 of –30.5 kJ/mol this makes the overall reaction energetically favorable, with a ΔG^0 of (–30.5 + 13.8) kJ/mol = –16.7 kJ mol:

			ΔG^0
glucose + phosphate	⇌	glucose-6-phosphate + H_2O	+13.8 kJ/mol
ATP + H_2O	⇌	ADP + phosphate	–30.5 kJ/mol
glucose + ATP	⇌	glucose 6-phosphate + ADP	–16.7 kJ mol

The coupling of both reactions is made possible by the enzyme hexokinase, which has two substrate binding sites, one for glucose and one for ATP, that bring both substrates into close proximity and orient them relative to each other such that the phosphate transfer reaction can be accelerated efficiently in the active center of the enzyme. Indeed, ATP hydrolysis is the main energy source of the cell for driving energetically unfavorable steps in biosynthetic pathways. ATP is however not the only phosphorylated compound used pro produce energy. Another important example is creatine phosphate, which even yields –43.1 kJ/mol upon hydrolysis. This means that phosphorylation of ADP by creatine phosphate to creatine and ATP is energetically favorable and can be used to synthesize ATP from ADP. The reaction is catalyzed by creatine kinase. Other examples of phosphorylated high-energy compounds are phosphoenolpyruvate and 1,3-bisphosphoglycerate, which yield –61.9 kJ/mol and –49.4 kJ/mol upon hydrolysis, respectively.

4.2. Removing an energetically unfavorable state of a molecule from equilibrium

The fourth step in glycolysis is the conversion of the six-carbon sugar fructose 1,6 bisphosphate to the three-carbon units dihydroxyacetone phosphate and glyceraldehyde 3-phosphate. Both thee-carbon units have the same elemental formula and are in a fast equilibrium with each other in the presence of triose phosphate isomerase. This enzyme catalyzes the attainment of the isomerization equilibrium, which is far on the side of dihydroxyacetone phosphate (95% dihydroxyacetone phosphate and 5% glyceraldehyde 3-phosphate). However, only the less stable glyceraldehyde 3-phosphate can undergo the next step of glycolysis, namely the oxidation of glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate, a reaction catalyzed by glyceraldehyde 3-phosphate dehydrogenase. Nevertheless, all molecules of dihydroxyacetone phosphate are converted to 1,3-bisphosphoglycerate: The reaction of glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate removes glyceraldehyde 3-phosphate from the equilibrium with dihydroxyacetone phosphate, and the equilibrium can only be attained again by formation of more glyceraldehyde 3-phosphate from dihydroxyacetone phosphate. This continues until eventually the entire fructose 1,6 bisphosphate is transformed to 1,3-bisphosphoglycerate.