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The relative values of the turnover number and the dissociation rate constant determine the definition of the Michaelis-constant

Gassan Nazzal

Independent author
E.mail address : gassannazzal@hotmail.com

Abstract . *The K_m value is widely used and defined as a measure of the stability of the ES-complex , however, this is true if the first reaction step exists in rapid equilibrium and the reaction is activation controlled . This paper will discuss the theoretical limitation of each case of the quasi-steady-state approximation using the statistical thermodynamic and the diffusion theory to define the K_m value .*

Keywords: K_m value, turnover number, Rapid equilibrium approximation, diffusion limited reaction, enzymatic efficiency, Transition state affinity.

1. Introduction

The main function of enzymes is to accelerate the reaction rate through destabilizing the substrate structure by a certain reaction mechanism, increasing in this manner the probability that the substrate will reach the transition state. Michaelis and Menten applied the rapid equilibrium approximation (REA) on the hydrolysis of sucrose by the enzyme Invertase , in the first step the enzyme binds the substrate reversibly, in the second step, the irreversible reaction takes place [1]

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

k_1 : The association rate constant of ES – complex ($M^{-1}s^{-1}$) .

k_{-1} : The unproductive dissociation rate constant of ES – complex (s^{-1}) .

k_{cat} : The turnover number (s^{-1}) .

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The REA states that the dissociation rate constant k_{-1} is fast in comparison with the turnover number k_{cat} . Thus, we can assume that the substrate concentration at the half maximal velocity is equal to the dissociation constant K_d , which indicates the affinity between E and S . This assumption is a special case of the quasi-steady-state approximation (QSSA) proposed by G. Briggs and J. Haldane in 1925 [2] . The QSSA defines the K_m value as :

$$K_m = \frac{k_{-1} + k_{cat}}{k_1} \qquad (1)$$

The REA is applicable if $k_{-1} >> k_{cat}$:

$$K_m = \frac{k_{-1} + k_{cat}}{k_1} \approx \frac{k_{-1}}{k_1} = K_d = \frac{[S]([E_0] - [ES])}{[ES]}$$

The second special case of the QSSA is the Van Slyke-Cullen mechanism[3] , where both steps are irreversible and $k_{-1} << k_{cat}$:

$$K_m \approx \frac{k_{cat}}{k_1}$$

The efficiency of the enzyme provides information about the ratio between k_{-1} and k_{cat} . If we assume that the association step is diffusion controlled thus we can assume that the association rate constant k_1 is almost equal to the diffusion rate of the molecules and The enzyme efficiency can be determined by comparing the k_{cat}/K_m value with the diffusion limit k_1 of a bimolecular reaction . :

$$Enzyme\ efficiency = \frac{k_{cat}}{K_m\, k_1} = \frac{k_{cat}}{k_{cat} + k_{-1}}$$

In this work we will determine the assumptions of each case .To do so we have to study the reaction rate constants, that defines the K_{m-} value to estimate an equation that predicts the enzyme efficiency .

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2 - The kinetics of the saturation behavior

Before the reaction takes place the molecules have to diffuse and collide with each other and only the collisions in the right point on the surface of the enzyme and with the sufficient amount of energy (activation energy E_a) will lead to the formation of the product. The sequence of one enzymatic cycle can be divided into two steps, namely the effective diffusion time to form an activated ES-complex and the duration of the maximal catalytic rate :

$$t_R = n.t_c + \frac{1}{k_{cat}} \qquad (2)$$

t_R : the duration of one enzymatic cycle.
n : denotes the total number of S molecules that are needed to hit the active site every cycle, until an energetically active molecule binds to form a product . We will define this parameter as the collision number .
t_C : diffusion -collision time , time until E and S encounter each other .

Assuming that all molecules are mixed and distributed equally in the solution, we can calculate the needed time to form an ES complex as a function of the substrate concentration, using the mean squared displacement equation. Firstly we define the diffusion -collision time as :

$$t_C = \frac{1}{f_c} \qquad (3)$$

The collision frequency can be expressed as follows:

$$f_c = \frac{molecular\ steps}{Diffusion\ time} \,.\, relative\ cross\ section \qquad (4)$$

We define one molecular step as the distance at which the molecule translocates its own diameter (2r). To diffuse a distance x, the molecule had to diffuse x/2r molecular steps, and for two molecules the number of steps will be :

$$molecular\ steps = \frac{x}{2(r_E + r_S)} \qquad (5)$$

The relative reaction cross section is the ratio between the targets area of a collision event and the total available area . In this case :

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$$relative\ cross\ section = \frac{\pi(r_E + r_S)^2}{\pi \frac{x^2}{4}} = \frac{4(r_E + r_S)^2}{x^2} \qquad (6)$$

To estimate the diffusion distance x, we assume that the substrate molecules in the solution are equally distributed in the total volume V . Thus, each substrate molecule occupies the volume V/N_S (divided by 1000 to convert from liter to m^3), N_A denotes the Avogadro's number :

$$\frac{V}{10^3 N_S} = \frac{1}{10^3 N_A[S]} = \frac{4\pi R^3}{3}$$

We define the diffusion distance as the diameter of the spherical volume per substrate molecule:

$$x = 2R = 2\,.\sqrt[3]{\frac{3}{4.10^3 \pi N_A[S]}} \qquad (7)$$

To calculate the diffusion time for two-body problem, we take the sum of the diffusion velocities of both molecules E and S, using the equation of the mean squared displacement for a three-dimensional case ($t = x^2 / 6D$) :

$$v_{ES} = \frac{dx}{dt} = v_E + v_S = 6\,(D_S + D_E)\,\frac{1}{x}$$

We assume that the substrate molecule diffuse the total volume per molecule until the collision occurs thus we can estimate the integral with the following boundary conditions to calculate the diffusion time :

$$\int_0^{2R} x dx = 6\,(D_S + D_E)\int_0^{t_D} dt$$

$$t_D = \frac{R^2}{3(D_S + D_E)} \qquad (8)$$

Substituting eq (5), (6) ,(7) and (8) in eq (4) we obtain :

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$$f_c = \frac{3(D_S + D_E)(r_E + r_S)}{R^3} = 4x10^3 \pi N_A (D_E + D_S)(r_E + r_S)[S]$$

Substituting the Stokes-Einstein– relation in the last equation we obtain equation (9) :

$$D_i = \frac{k_b T}{6\pi r_i \eta}$$

$$f_c = \frac{1}{t_c} = \frac{2}{3} \cdot \frac{10^3 [S] RT}{\eta} \frac{(r_E + r_S)^2}{r_E . r_S} \qquad (9)$$

f_c : the collision frequency at a given substrate concentration (s^{-1}).
η : denotes the dynamic viscosity ($N.s.m^{-2}$) .
r_E and r_s the radius of each molecule (m) .
[S] the molar substrate concentration .

One of the limitations of the equation (9) is that the substrate concentration will decrease with the time as a result of the enzymatic activity . Consequently, the collision frequency is time-dependent .Therefore, we must adjust that $[S]_0 >> [E]_0$ to assume the free ligand approximation $[S]_t \approx [S]_0$.

The reaction rate can be described, as the catalytic frequency f_{cat} (s^{-1}) of one enzyme molecule multiplied with the total enzyme concentration in molar units $[E_0]$:

$$\frac{1}{f_{cat}} = n.t_c + \frac{1}{k_{cat}} = \frac{n}{f_c} + \frac{1}{k_{cat}} \qquad (11)$$

$$v = f_{cat}[E_0] = \frac{[E_0]}{\frac{n}{f_c} + \frac{1}{k_{cat}}} \qquad (12)$$

Rearranging the Michaelis-Menten equation in a similar form as equation (12) , we obtain :

.

$$v = \frac{k_{cat}[E_0][S]}{\frac{k_{cat} + k_{-1}}{k_1} + [S]} = \frac{[E_0]}{\left(\frac{k_{cat} + k_{-1}}{k_{cat}}\right)\frac{1}{k_1[S]} + \frac{1}{k_{cat}}} \qquad (13)$$

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Equating equation (12) and (13) :

$$f_{cat} = \frac{1}{\frac{n}{f_c} + \frac{1}{k_{cat}}} = \frac{1}{\left(\frac{k_{cat} + k_{-1}}{k_{cat}}\right)\frac{1}{k_1[S]} + \frac{1}{k_{cat}}} \qquad (14)$$

Equation (14) represents a redefinition of the Km as the equivalence point between the effective diffusion rate and the maximal reaction rate , rather than the classical concept of the enzyme saturation that describes a general protein- ligand interaction.

At [S] = K_m ,the effective diffusion rate, and the maximal reaction rate are equivalent and we obtain an expression which is similar to the Arrhenius equation :

$$at : [S] = K_m \ : \ v = \frac{k_{cat}[E]_0}{2} = \frac{[E_0]}{\frac{n}{f_c} + \frac{1}{k_{cat}}} = \frac{[E_0]}{\left(\frac{k_{cat} + k_{-1}}{k_{cat}}\right)\frac{1}{k_1 K_m} + \frac{1}{k_{cat}}}$$

Thus :

$$\frac{n}{f_c} = \frac{1}{k_{cat}} = \left(\frac{k_{cat} + k_{-1}}{k_{cat}}\right)\frac{1}{k_1 K_m}$$

$$k_{cat} = k_1 K_m \left(\frac{k_{cat}}{k_{cat} + k_{-1}}\right) = \frac{2}{3}.\frac{10^3 K_m RT}{\eta}\frac{(r_E + r_S)^2}{r_E.r_S}.\frac{1}{n} \qquad (15)$$

The diffusion controlled bimolecular rate constant of the ES- complex formation can be theoretically calculated as :

$$k_1 = \frac{f_c}{K_m} = \frac{2.10^3 RT}{3\eta}\frac{(r_E + r_S)^2}{r_E.r_S} \qquad (16)$$

The total maximal collision frequency when [S] = K_m is equal to :

$$f_c = k_1 K_m = k_{cat} + k_{-1} \qquad (17)$$

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3. The energy profile of the enzyme action

The K_m value is the sum of the dissociation constant K_d and the effective substrate concentration (see eq 10) .Mathematical expression of this statement can be written through substituting equations (15) and (16) in equation (20) and then in equation (1) we obtain the equation (21) :

$$K_d = \frac{k_{-1}}{k_1} \qquad (19)$$

$$\frac{k_{cat}}{k_1} = K_m . \frac{1}{n} \qquad (20)$$

$$K_m = K_d . \left(\frac{n}{n - 1} \right) \qquad (21)$$

3.1 The collision number determines the probability of the reaction :

Rearranging the K_m expression into two fractions allow us to treat the system as a canonical ensemble of the ES-complex . The two fractions denote the probabilities of two dissociation events ,namely , the event that the complex will stay in association-dissociation- equilibrium with the free substrate and the free enzyme and the event that the complex will reach the transition state and react :

$$\frac{k_{cat}}{k_1 K_m} + \frac{k_{-1}}{k_1 K_m} = 1 \qquad (22)$$

The Boltzmann distribution allows us to relate the fraction of molecules in a certain state to the probability to find this state :

$$p_i = \frac{N_i}{N} = \frac{number\ of\ molecules\ in\ state\ i}{total\ number\ of\ the\ molecules} = \frac{1}{\sum e^{-\frac{E_i}{k_b T}}} e^{-\frac{E_i}{k_b T}}$$

P_i : the probability of the state i in the system .
E_i : The energy level of the state i .
k_b : The Boltzmann constant , T : The absolute temperature .

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In one catalytic cycle, one substrate molecule will be converted to a product .In one second the productivity is equal to the turnover number which indicates the successful dissociation of the ES-complex after reaching the transition state .The probability $p_{\ddagger}$ to find an ES-complex in the transition state with the energy level $E^{\ddagger}$ is :

$$p_{\ddagger} = \frac{1}{\sum e^{-\frac{E_i}{k_b T}}} e^{-\frac{E^{\ddagger}}{k_b T}} = \frac{k_{cat}}{k_1 K_m} = \frac{1}{n} \qquad (23)$$

On the other hand (n - 1) ES molecules are in equilibrium with the free form of the enzyme and the substrate. Assuming that the association event is diffusion controlled , the dissociation event is under thermodynamic control and had to overcome the dissociation energy . The probability p_0 to find an ES-complex in the unproductive dissociated form is *:*

$$p_0 = \frac{1}{\sum e^{-\frac{E_i}{k_b T}}} . e^{-\frac{(E_E^0 + E_S^0)}{k_b T}} = \frac{k_{-1}}{k_1 K_m} = \frac{K_d}{K_m} = \frac{n-1}{n} \qquad (24)$$

To simplify the calculations we use the activation energy $\Delta E^{\ddagger}$, and the dissociation energy of the ES-complex ΔE_{diss} , E^0_{ES} denotes the ground state of the ES-complex :

$$E^{\ddagger} = \Delta E^{\ddagger} + E_{ES}^0 \qquad (25)$$

$$E_E^0 + E_S^0 = \Delta E_{diss} + E_{ES}^0 \qquad (26)$$

Substituting eq(25) in eq(23) and eq(26) in eq(24) and then estimating the ratio $p_{\ddagger}/p_0$ to determine the collision number results in the relation :

$$\frac{p_{\ddagger}}{p_0} = e^{-\frac{(\Delta E^{\ddagger} - \Delta E_{diss})}{k_b T}} = \frac{1}{n-1} = \frac{k_{cat}}{k_{-1}}$$

$$n = 1 + e^{\frac{\Delta E^{\ddagger} - \Delta E_{diss}}{k_b T}}$$

Thus, the enzyme efficiency is the probability that the ES-complex will reach the transition state . The probability of the catalytic event and the collision number are determined by the difference between the dissociation energy barrier and the activation energy barrier :

$$p_{\ddagger} = \frac{1}{n} = \frac{e^{-\frac{\Delta E^{\ddagger}}{k_b T}}}{e^{-\frac{\Delta E_{diss}}{k_b T}} + e^{-\frac{\Delta E^{\ddagger}}{k_b T}}} = \frac{k_{cat}}{k_{cat} + k_{-1}} \qquad (28)$$

When $\Delta E^{\ddagger} >> \Delta E_{diss}$, then we obtain the expression of the Arrhenius equation of the activation energy E_a :

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$$E_a = E^{\ddagger} - (E_E^0 + E_S^0)$$

$$p_{\ddagger} = e^{-\left(\frac{\Delta E^{\ddagger} - \Delta E_{diss}}{k_b T}\right)} = e^{-\frac{E_a}{k_b T}}$$

The ratio between K_d / K_m is the probability that the ES-complex is in association –dissociation equilibrium:

$$p_0 = \frac{e^{-\frac{\Delta E_{diss}}{k_b T}}}{e^{-\frac{\Delta E_{diss}}{k_b T}} + e^{-\frac{\Delta E^{\ddagger}}{k_b T}}} = \frac{n-1}{n} \qquad (29)$$

The ratio K_m / K_d can be evaluated as a vibrational partition function relative to the ground states of the free enzyme and the free substrate :

$$\frac{K_m}{K_d} = \frac{1}{1-\frac{1}{n}} = 1 + e^{-\frac{(\Delta E^{\ddagger} - \Delta E_{diss})}{k_b T}} = 1 + e^{-\frac{E_a}{k_b T}}$$

The evaluated partition function represents the ability of the catalytic reaction mechanism to destabilize the ground state of the substrate, populating in this way the transition state . When the partition function equals 1 , this means that all the ES-molecules exist in the ground state and the active site exhibits no catalytic activity , therefore the system exists in association – dissociation equilibrium $K_m = K_d$.

3.2 The affinity toward the transition state determines the probability of the reaction :

Using the Eyring-equation we can express the equation (1) in terms of free energy. For zero order reaction the relation between the rate constant and the free energy of the activation $\Delta G^{\ddagger}$ is :

$$k = k_{cat} = \frac{k_b T}{h} e^{-\frac{\Delta G^{\ddagger}}{RT}} \qquad (30)$$

In equation (30) the transmission coefficient is assumed to equal 1.

$$K_m = K_d + \frac{k_{cat}}{k_1} = K_d + \frac{k_b T}{k_1 h} e^{-\frac{\Delta G^{\ddagger}}{RT}} = K_d \cdot \left(\frac{n}{n-1}\right)$$

Substituting the relation between the dissociation constant and the free energy of the binding ΔG_b we obtain :

$$n = 1 + \frac{1}{\frac{k_b T}{k_1 h} e^{-\frac{(\Delta G^{\ddagger} + \Delta G_b)}{RT}}} \qquad (31)$$

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We can now express the probability of the catalytic event in terms of free energies using equation (31)

$$p_{\ddagger} = \frac{1}{n} = \frac{\frac{k_b T}{k_1 h}\, e^{-\frac{(\Delta G^{\ddagger}+\Delta G_b)}{RT}}}{1 + \frac{k_b T}{k_1 h}\, e^{-\frac{(\Delta G^{\ddagger}+\Delta G_b)}{RT}}}$$

The Eyring theory proposed that the reactant and the transition state are in rapid equilibrium state , thus we can express the equilibrium constant of the transition as :

$$K^{\ddagger} = e^{-\frac{\Delta G^{\ddagger}}{RT}} = \frac{[ES^{\ddagger}]}{[ES]} = \frac{K_d k_1 h}{k_b T}\,\frac{p_{\ddagger}}{1 - p_{\ddagger}}$$

The ratio $K^{\ddagger}/K_d$ represents the affinity of the enzyme toward the transition state :

$$\frac{K^{\ddagger}}{K_d} = e^{-\frac{(\Delta G^{\ddagger}+\Delta G_b)}{RT}} = \frac{[ES^{\ddagger}]}{[E][S]}$$

$$p_{\ddagger} = \frac{\frac{k_b T}{k_1 h}}{\frac{K_d}{K^{\ddagger}} + \frac{k_b T}{k_1 h}}$$

The ratio $K^{\ddagger}/K_d$ represents the ability of the active site firstly to recognize the right substrate and secondly to destabilize it . The sum of the free energy differences of both steps indicates that the two enzymatic steps are coupled , the binding step is exergonic while the destabilizing step is endergonic . thus, we can assume that the substrate binding induces a conformational change that is complementary to the transition state . The probability to induce a conformational change that opens the reaction path is maximized when the sum of the free energies differences of the ground state and the transition state formation is minimized. This is a conformational proofreading mechanism [4] , the substrate binding induces a conformational change that causes a deformation in the substrate structure , hence, the lower the affinity toward the substrate the higher the unproductive dissociation of the transition state .

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4. Defining the assumptions of each case of the QSSA

We can now determine the conditions of the major cases:

1-Activation controlled enzyme :The energy difference between the activation energy and the dissociation energy is high ,thus the catalytic event is unlikely to proceed and the active site exhibits a low stabilizing ability of the transition state . The majority of the association events dissociate unproductively and the system exists in a rapid equilibrium state :

$$\Delta E^{\ddagger} - \Delta E_{diss} >> 0$$

$$p_{\ddagger} << p_0$$

$$\frac{K_d}{K^{\ddagger}} >> \frac{k_b T}{k_1 h}$$

$$K_m \approx K_d$$

$$k_{cat} << k_{-1}$$

$$\frac{k_{cat}}{k_1 K_m} << \frac{1}{2}$$

The validity of the REA : The average enzyme exhibits a k_{cat} of 10 s^{-1} and a K_m value of 100 µM [5] . Taking the theoretical diffusion limit as 10^9 $M^{-1}s^{-1}$ to calculate the ratio (k_{cat}/ K_m) / k_1 will yield an enzyme efficiency of 0.01% , which is equal to the relative deviation between K_m and K_d .

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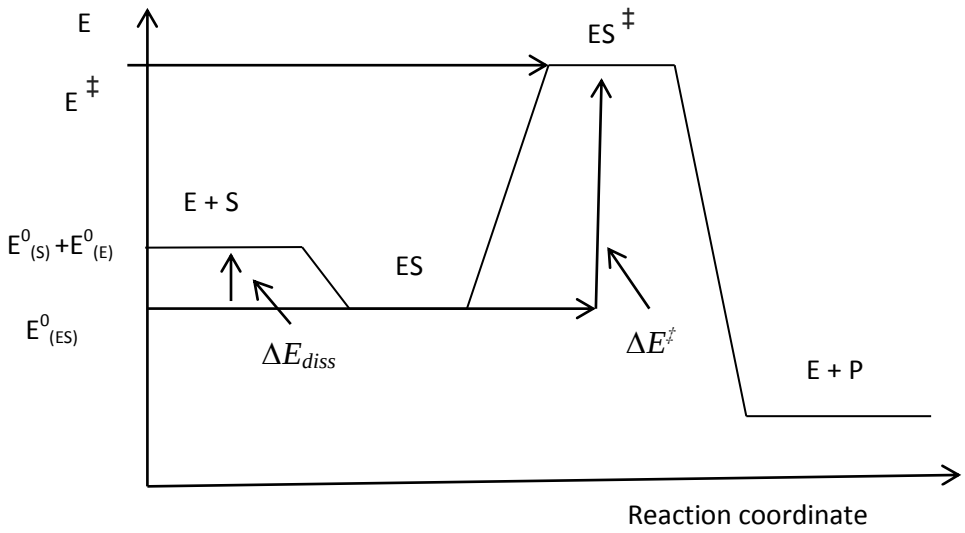

Figure 1 Case 1 the difference between the activation energy and the dissociation energy is high thus the majority of the molecules are in rapid association-dissociation equilibrium.

2- The energy difference between the activation energy and the dissociation energy equals zero, therefore, the ES-complex dissociates equally in both directions and the enzymatic efficiency is equal to 50% of the effective collisions . The ES-complex will dissociate in both directions equally the reaction is diffusion controlled :

$$\Delta E^{\ddagger} - \Delta E_{diss} = 0$$

$$p_{\ddagger} = p_0 = \frac{1}{2}$$

$$\frac{K_d}{K^{\ddagger}} = \frac{k_b T}{k_1 h}$$

$$K_m = 2K_d$$

$$k_{cat} = k_{-1}$$

$$\frac{k_{cat}}{k_1 K_m} = \frac{1}{2}$$

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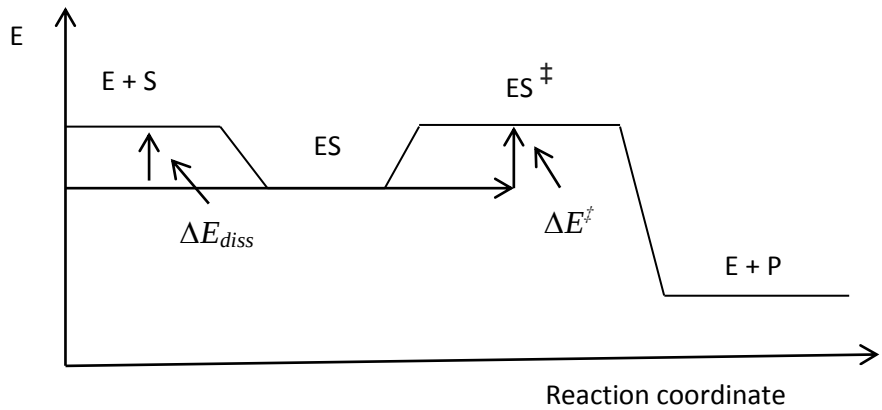

Figure 2 case 2 the activation energy and the dissociation energy are equal thus, the probabilities of the dissociation are equally probable in both directions .

3- Diffusion-controlled enzyme : The activation energy barrier is lower than the dissociation energy thus the dissociation is directed forward irreversibly in a way that nearly every collision leads to a reaction:

$$\Delta E^{\ddagger} - \Delta E_{diss} < 0$$

$$p_{\ddagger} >> p_0$$

$$\frac{K_d}{K^{\ddagger}} << \frac{k_b T}{k_1 h}$$

$$K_m \approx \frac{k_{cat}}{k_1}$$

$$k_{cat} >> k_{-1}$$

$$\frac{k_{cat}}{k_1 K_m} \approx 1$$

The difference between the activation energy and the dissociation energy becomes negative if the reaction proceeds stepwise each step had a relatively low activation energy barrier to overcome and the released energy from the first reaction step makes the total difference ΔΔE more negative.

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- The productive dissociation constant k_{cat}/k_1 :

In the literature, the value of k_{cat}/K_m is widely used as a measure of the enzyme efficiency because it can be easily compared with the diffusion controlled association rate constant k_1 of a bimolecular reaction . In contrast to k_{cat}/K_m the ratio k_{cat}/k_1 represents the substrate concentration that could be converted to products at half-maximal reaction rate :

$$\frac{k_{cat}}{k_1} = K_m p_{\ddagger}$$

To understand the reason why some enzymes work kinetically perfect and other not , we need to consider the biological context of the enzyme kinetics .An activation controlled enzyme may be kinetically inefficient but in vivo, this inefficiency may be very important for the cellular homeostasis and the biochemical control . Assuming that the ratio k_{cat}/k_1 is the wanted physiological concentration of the product in the cell ,increasing the efficiency will lead to accumulation of the product that may distort the homeostasis bringing the organism into a pathological state . The substrate consumption may be shared by two or several pathways , increasing the efficiency of one enzyme in one pathway will lead to a higher consumption of the substrate in the kinetically more efficient pathway which may cause a deficiency in the product formation in the less efficient pathway .Biological pathways are in many cases regulated. Hypothetically if every enzyme in the cell would be diffusion controlled the organism will need additional regulating mechanisms to maintain the concentrations of the products and the substrates in the physiological range, because the enzyme accelerates the achievement of the equilibrium state which is not the physiological state in most cases,and this will consume energy and the system will be more vulnerable to the external factors. Thus, the pre-defined enzyme efficiency will yield a defined product formation without the need to add more regulatory points making the biochemical map more complex. Hence, the activation energy from the biological perspective is a required protective barrier to maintain the stability of the cellular structure and function . A high efficiency would be beneficial in the elimination of toxic compounds to protect the cell . A diffusion-controlled enzyme will eliminate a specific cytotoxic compound in every encounter examples : catalase , superoxide dismutase , β-lactamase [7] . Another biological function that needs a total elimination of the substrate would be the switch of a biological function between on or off mode , a typical example is the acetylcholine -esterase .rapid total elimination of the neurotransmitter from the synaptic cleft decreases the regeneration time making the system ready to sense a new signal.

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5. Theoretical calculations

$p_{\ddagger}$	0.99	0.8	0.5	0.2	10^{-1}	10^{-2}	10^{-4}	10^{-6}
n	1.01	1.25	2	5	10	100	10^{4}	10^{6}
$\Delta\Delta E$ kJ/mol	-11.382	-3.433	0	3.433	5.442	11.38	22.813	34.22
K_d µM	1	20	50	80	90	99	99.99	99.999
$K^{\ddagger}$	$1.04x10^{-7}$	$8.48x10^{-8}$	$5.3x10^{-8}$	$2.12x10^{-8}$	$1.06x10^{-8}$	$1.06x10^{-9}$	$1.06x10^{-11}$	$1.06x10^{-13}$
$K^{\ddagger}/K_d$ M^{-1}	0.104	$4.24x10^{-3}$	$1.06x10^{-3}$	$2.65x10^{-4}$	$1.17x10^{-4}$	$1.07x10^{-5}$	$1.06x10^{-7}$	$1.05x10^{-9}$
ΔG_b kJ/mol	-34.221	-26.800	-24.530	-23.366	-23.074	-22.838	-22.814	-22.814
$\Delta G^{\ddagger}$ kJ/mol	39.827	40.332	41.497	43.766	45.483	51.187	62.594	74.024
k_{cat}/k_1 µM	98	80	50	20	10	1	0.01	0.0001
k_{-1} s^{-1}	$6.6x10^{3}$	$1.32x10^{5}$	$3.3x10^{5}$	$5.28x10^{5}$	$5.49\ x10^{5}$	$6.53x10^{5}$	$6.59\ x10^{5}$	$6.59\ x10^{5}$
k_{cat} s^{-1}	$6.53x10^{5}$	$5.28x10^{5}$	$3.3x10^{5}$	$1.32x10^{5}$	$6.6\ x10^{4}$	$6.6\ x10^{3}$	66	0.66
k_{cat}/K_m $M^{-1}s^{-1}$	$6.53x10^{9}$	$5.28x10^{9}$	$3.3x10^{9}$	$1.32x10^{9}$	$6.6\ x10^{8}$	$6.6\ x10^{7}$	$6.6\ x10^{5}$	$6.6\ x10^{3}$

Table 1 : Theoretical calculations : we have maximized theoretically the enzyme efficiency $p_{\ddagger}$ at constant K_m value , K_m = 100 µM . The diffusion limit is calculated by assuming that the substrate and the enzyme are in the same size $r_E = r_S$, temperature at 25 ºC , and the dynamic viscosity is equal to 1 mPa.s .

$$f_c = 10^3 x10^{-4} \left[\frac{mol}{m^3}\right] \frac{8x8.314\left[\frac{J}{molxK}\right] x298[K]}{3x\ 10^{-3}\left[\frac{N.s}{m^2}\right]} \approx 6.6\ x10^5\ s^{-1}$$

$$k_1 = \frac{f_c}{K_m} = \frac{6.6\ x10^5\ [s^{-1}]}{10^{-4}\ [M]} = 6.6\ x\ 10^9\ M^{-1}s^{-1}$$

$$k_{cat} = 6.6\ x10^5\ x\ p_{\ddagger} \qquad , \qquad k_{-1} = 6.6\ x10^5\ (1 - p_{\ddagger})$$

$$\Delta\Delta E = \Delta E^{\ddagger} - \Delta E_{diss} = 2.477\left[\frac{kJ}{mol}\right] ln\frac{1 - p_{\ddagger}}{p_{\ddagger}}$$

$$K_d = K_m.(1 - p_{\ddagger}) = e^{\frac{\Delta G_b}{2.477}}$$

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$$K^{\ddagger} = \frac{6.6\,x\,10^{9}\,[\,M^{-1}s^{-1}]x6.626x10^{-34}[Jxs]}{1.38x10^{-23}\left[\frac{J}{K}\right]x298[K]}\frac{p_{\ddagger}}{1-p_{\ddagger}}.K_d = 1.06x10^{-3}[M^{-1}]\frac{p_{\ddagger}}{1-p_{\ddagger}}.K_d$$

4. Conclusion

In this work, we attempt to determine the assumptions of each case of the QSSA. We came to the conclusion that for an enzyme with average kinetics parameters the REA is a good approximation to derive the rate equation and the K_m value tends to equal the dissociation constant K_d .In contrast, when the enzyme is diffusion controlled, the energy level of the transition state is equal or lower than the energy levels of the free reactants thus the K_m value is equal to the productive dissociation rate k_{cat}/k_1 . The reaction mechanism of the active site splits or classifies the population of the substrate into two energy states, the ground state, and the transition state. The ratio K_m/K_d is equal to the vibrational partition function of the assumed two-state-system. For the average enzyme , the partition function of the transition tends to equal 1 thus the majority of the substrate molecules are in the ground state and the assumption $k_{cat} << k_{-1}$ is valid hence $K_m \approx K_d$ .We have redefined the K_m value as the equivalence point of the reaction rates, namely, the effective diffusion rate and the maximal catalytic rate, which reflects more clearly the transition from the bimolecular reaction to the unimolecular reaction in the saturation curve .

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