International Journal of Biochemistry Research & Review
15(2): 1-12, 2016; Article no.IJBCRR.31203
ISSN: 2231-086X, NLM ID: 101654445

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Space-time and Entropic Characterization of *Aspergillus oryzea* α-amylase Catalyzed Reaction

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Author's contribution

The sole author designed, analyzed and interpreted and prepared the manuscript.

Article Information

DOI: 10.9734/IJBCRR/2016/31203
Editor(s):
(1) Yi-Ren Hong, College of Medicine, Kaohsiung Medical University, Taiwan.
Reviewers:
(1) Eliton da Silva Vasconcelos, Federal University of São Carlos – UFSCAR, Brazil.
(2) Ayhan Goktepe, Selcuk University, Konya, Turkey.
Complete Peer review History: http://www.sciencedomain.org/review-history/17622

Original Research Article

Received 26th December 2016
Accepted 23rd January 2017
Published 26th January 2017

ABSTRACT

Aims: The aims of this investigation were to determine: 1) the distribution of time and time-dependent events in a catalytic cycle, 2) maximum and minimum (average) interparticle distance between interacting molecules, 3) quantify the entropic term, and 4) elucidate the implication of entropic "barrier".

Methodology: The study design is theoretical and experimental. The place of research was Research Division of Ude International Concepts Limited (RC 862217), B.B. Agbor and Owa Alizomor Sec. Sch. Owa Alizomor, Delta State, Nigeria. The research spanned a period of ~ 1.5 years with stoppages between 20-07-2015 and 02-12-2016. Bernfeld method of enzyme assay was used. Data generated from assay of enzyme and calculated data were fitted to the models to determine the desired parameters.

Results: The catalytic orientation time ranging from 4.78-43.78 μs corresponds to value of [S] ranging from 22.04 g/l-3 g/l. Average time for the formation of product per molecule of enzyme, $1/\langle k_2 \rangle$ = 2.58 ms. Translational entropy, TE = 28.95±0.03 J/mol.K. The entropy of dilution, $\Delta S_{\infty dil}$, at infinite dilution = 64.75±1.51 J/mol.K. TE (TE_∞) when t_c = 0, is = 6.27±1.13 kJ/mol.K. The minimum interparticle distance at maximum concentration of substrate, l_m = 5.3142±0.06 exp (-8) m.

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Conclusion: Different aspects of catalytic action include transit before effective collision, catalytic orientation, catalytic transformation of product and product release. Each aspect has its duration. The time (t_c) spent in catalytic transformation of substrate and release of product is < the time (reciprocal of rate constant, $1/k_2$) taken to yield a product by a molecule of an enzyme. ($1/\langle k_2 \rangle$) - $\langle t_c \rangle$ = catalytic orientation time. Period of transit is « period within the active site. TE, unlike entropic "barrier" due to the state of dilution, promotes catalytic function.

Keywords: Aspergillus oryzea alpha amylase; translational entropy; entropic "barrier"; catalytic cycle; time; dilution.

1. INTRODUCTION

It is a well known observation that solutes, dissolved or undissolved, are subject to solvent bombardment on account of which they are involved in random motion. Thus before hitting a target the bullet molecule must have moved in different direction thereby making the distance between the bullet and target to appear longer than what should have been the case. Some researchers may mix the reaction mixture component by swirling or shaking the test tube containing reaction mixture. It may be strange to suggest that mechanical mixing only aids the distribution of reaction mixture component. In other words frequency of collision does not necessarily increase substantially because each molecule is subject to the force generated from mechanical mixing. Each molecule is compelled to a new position just as another molecule is forced to take the place of displaced molecule. However, the focus has always been on diffusion, a process that covers three-dimensional space (3-D), by which macromolecules and nanoparticles in the extracellular matrix (ECM) is crucially involved in the delivery of therapeutic agents in tumour tissues [1]. Yet, experimental findings have shown that diffusion can be significantly hindered by electrostatic interactions between the diffusing particles and charged components [1]. This observation is relevant to enzyme-substrate interaction facilitated by eventual mutual electrostatic attraction that imposes unidirectionality on the interacting molecules as expressed in submitted manuscript.

Translational diffusion or velocity is temperature dependent and therefore, there must be an entropic term best described as translational entropy (TE) with different connotation in accordance with the context in which it is applied: There is TE gain upon formation of a macromolecular complex [2] and for the folding of proteins [3] with different methods of determination. Experimentation by comparing the entropy of unfolding/dissociation of the complex with the entropy of its unfolding without dissociation i.e. unfolding of the same complex having covalently linked subunits [2] is another approach. The 3D integral equation theory which is an extension to general systems described using the x-y-z coordinate system (an approach not understood by the author of this work) is employed for the formulation of the model for the determination of TE [3]. Using classical statistical mechanics, researchers [4] express "the translational entropy S_{ξ}^{trsl} of a given concentration of solute, ξ in solution (i.e., N_{ξ} solute and N_W solvent molecules in a volume, V at a temperature, T.), in terms of the molecular partition function of one solute, q_{ξ}". Once again this entails very high mathematical formalism. In this research a method with simple mathematics which depends in part on kinetic parameter and consequently activity of the enzyme is formulated and applied. The model is premised on the fact that no contact between the enzyme and the substrate can be made without translational velocity, and consequently translational entropy. The model proposed is applicable to biological system.

It has also been observed that reaction rate always decreases as the volume fraction of crowding agents is increased due to the reduced diffusion coefficient [5] as may also be applicable to intracellular medium. The situation may not be exactly the same in extracellular medium and purely reaction mixture solution in test tubes. However, collision may occur between substrate and enzyme at sites other than active site leading to delay in complex formation. The important issue is that time and space ultimately determines the likelihood of the occurrence of enzymatic transformation of substrate to product or the formation drug-poison/pathogen complex before therapeutic transformation. The total time involved in the transformation of substrate could be determined if individual enzyme molecule is

handled separately. Incidentally it has been observed that advancement in single-molecule spectroscopy have made it possible to follow the stochastic activity and, consequently, the catalytic properties of individual enzymes over extended period of time [6,7]. Although three dimensional structures of enzymes are not unusual, the consideration for a single enzyme molecular translational velocity/diffusion is nevertheless a one-dimensional diffusion [8] study. The total time it takes an enzyme to transform a substrate includes period of transit before contact with the substrate, period of binding, period of catalysis-breaking and making of bonds- and release of the product. This issue has been partly examined by Reuveni et al. [6]. Meanwhile, according to Reuveni et al. [6] the turnover rate (k_{turn}) has been expressed as: $k_{turn} = k_{cat}$ [S]/([S]+K_m) where [S], k_{cat} and K_m are the concentration of substrate, rate constant, and Michaelis-Menten constant respectively. But the former equation is not different from $v/[E]_T = k_2[S]/(K_m+[S])$ where v, $[E]_T$ and k_2 are the velocity of hydrolysis of starch, concentration of the enzyme and rate constant respectively, as may be obtainable from original Michaelis-Menten equation; $k_{turn} = 1/\langle T_{urn}\rangle$ where $\langle T_{urn}\rangle$ is the mean turnover time, the average time it takes a single enzyme to produce a single molecule of product [6]. The deduction to be made is that the reciprocal of k_{turn} (or $v/[E]_T$) shows that $\langle T_{urn}\rangle$ is always > the mean time for binding, catalysis, and product release, combined.

It takes time before contact between the enzyme and the substrate can be made. It also takes time before catalysis is completed. The space available to the enzyme molecule in its solution depends on its concentration. Mass diffusion occurs in 3-D space which is important for the distribution of particles within available space. But the linear component, the interparticle distance (being the length of the space between particles), is more important in the determination of the time it may take the enzyme to make catalytic contact with the substrate. It also takes some time for the breaking and making of bonds in the active site whose 3-D structure can accommodate the substrate. Therefore, any enzyme activity can be characterized in terms of time and space (or spatiotemporally) within and outside the active site. Therefore, the objectives of this investigation are to determine: 1) the distribution of time and time-dependent events in a catalytic cycle, 2) minimum interparticle distance 3) quantify the entropic term and 4) elucidate the implication of interparticle distance or entropic "barrier".

1.1 Theoretical Background

The following equation arises from Einstein – Smoluchowski equation:

$$l_x = (2Dt_x)^{1/2} \quad (1)$$

where t_x is the time spent outside the active site; D is the diffusion coefficient of the enzyme; l_x is the average intermolecular distance. Within the duration of assay in the presence of sufficient substrate molecules, the enzyme undergoes several cycles of catalytic activity (Φ). Given that the duration of assay is t_∞, the relationship between t_x and Φ is:

$$t_x = (t_\infty - \Phi t_c)/\Phi \quad (2)$$

where t_c is the time spent in the breaking and making of bond during the hydrolysis of glycosidic bond, and product release. Substitution of Eq. (2) into Eq. (1) gives:

$$l_x = \{2D(t_\infty - \Phi t_c)/\Phi\}^{1/2} \quad (3)$$

Making Φ subject of the formula gives:

$$\Phi = 2t_\infty D/(l_x^2 + 2Dt_c) \quad (4)$$

Thus the total time, t_{Tc}, spent in the active site of all participating enzyme molecules in bond breaking, bond making, and product release, within the duration of assay is expressed as:

$$t_{Tc} = 2t_\infty Dt_c/(l_x^2 + 2Dt_c) \quad (5)$$

The total time, $t_{\infty x}$, spent before complex formation within the duration of assay without substrate exhaustion is:

$$\begin{aligned} t_{\infty x} &= t_\infty - t_{Tc} \\ &= t_\infty - \{2t_\infty Dt_c/(l_x^2 + 2Dt_c)\} \end{aligned} \quad (6)$$

It must be made clear that the choice and use of l_x is intended to serve as root mean square displacement so as to reflect the original Einstein model $l_x^2/2D$ where l_x^2 is then the mean square displacement (the square of average interparticle distance) such that the time calculated based on the model should be merely average time.

Equation (4) can however, be restated as:

$$\Phi/t_\infty = 2D/(l_x^2 + 2Dt_c) = k_2 \quad (7)$$

where k_2 is the rate constant, otherwise called turnover number.

Equation (7) implies that the number of catalytic cycles divided by the duration of assay in the presence of sufficient substrate is k_2. The duration of hydrolytic action different from the combined duration of hydrolysis and duration of transit through the average interparticle distance can be obtained from Eq. (7) as follows.

$$(l_x^2 + 2Dt_c)/2D = 1/k_2 \quad (8)$$

Thus,

$$t_c = [2D - k_2 l_x^2]/2Dk_2 \quad (9)$$

The time spent in the active site during the breaking and making of new bond, and product release is expectedly < $t_c + t_x$. Thus the time spent outside the active site per molecule of the enzyme is expressed in Eq. (2). The total time spent within the core active sites is = $\Phi\, t_c$ and total time outside the active site while the enzyme is in transit towards the substrate is = $t_\infty - \Phi\, t_c$. Besides, it can be seen from Eq. (9) that: $l_c = 2Dt_c = [2D - k_2 l_x^2]/k_2$, that is, within the period, the complex is formed and undergoing transformation, the average distance covered by any free enzyme is l_c.

Equation (9) has important implications. As $l_x \rightarrow$ zero, $t_c \rightarrow 1/k_2$. If $2D > k_2 l_x^2$, the concentration of substrate [S] is approaching saturating concentration; if $k_2 l_x^2 > 2D$, then t_c should be negative with larger negative magnitude if $k_2 l_x^2 \gg 2D$. Off course if $2D = k_2 l_x^2$, then $t_c = 0$ because [S] $\rightarrow$ zero (or infinite dilution). It should be realized that if the time allocated for breaking of bond, making of bond, and product (P) realize is zero, then $\Delta P = 0$. It is obvious that when $2D/k_2 > l_x^2$, $t_c < 1/k_2$ and consequently, $1/t_c > k_2$. This must be understood in terms of t_c being just the time taken to break and make bond(s) and product release. The quantity t_c does not include transit time and catalytic orientation! Both space (conformational flexibility) and time are needed for the transformation of substrate within the active site. Therefore, Eq. (9) is important because it shows that catalytic process in the active site needs sufficient time. Transit time over the distant of closest approach of the enzyme to the substrate within the sphere of mutual interaction leading to complex formation and time for product departure is part of total time. If product does not depart, inhibition becomes inevitable. It is impossible for product to evolve by any means, biosynthetic or hydrolytic if t_c is negative or zero.

The implication of "$2D = k_2 l_x^2$" can be visualized from entropic perspective. Unlike thermodynamics of expansion of gases (in volume to be precise) at constant temperature, expressed in terms of entropy change (ΔS), less attention seem to be paid to ΔS implied in the dilution of a solution. Before the use of "$2D = k_2 l_x^2$" it is important to consider the equation put forward by Fitter [9].

The equation is:

$$\Delta S = 3R \ln (r_2/r_1) \quad (10)$$

where in Fitter's definition [9], r_2 and r_1 are different radii of a sphere; R is the usual gas constant. Thus, if

$$"2D = k_2 l_\infty^2" \quad (11)$$

$$l_\infty = (2D/k_2)^{1/2} \quad (12)$$

For the purpose of contrast, l_∞ is used in place of l_x. This distance may be much longer than interparticle distance (l_m) at which there may be mutual electrostatic or any other form of interaction leading to complex formation. It should therefore, be definitely longer than the given average interparticle distance at different concentration of the substrate. The value of l_x can be determined according to the equation: $l_x = (V/(\Sigma n)N_A)^{1/3}$ where V, Σn, and N_A are the volume of reaction mixture, sum of the number of moles of the substrate and enzyme, and Avogadro's number respectively. Such distance needs to be defined or formulated. However, experiment should be conducted to determine the value of l_m based on the fact that the closest distance of approach of the enzyme to the substrate is applicable where there is saturating concentration of the substrate [S] that results in maximum velocity (V_{max}) of hydrolysis of substrate when all the enzyme molecules are participating in enzyme-substrate complex formation. At velocity of hydrolysis < V_{max}, [ES]<[E_T], that is fewer number of enzyme molecules are involved in complex formation. But this does not preclude the fact that complex formation must begin at the same distance (l_m) of closest approach of the enzyme to the more massive substrate. Therefore, this interparticle distance, l_m in this case is applicable to any other velocity of hydrolysis of substrate even if [S] is less than Michaelis-Menten constant, K_m. In

order to achieve a stable enzyme-substrate complex suitable for transformation to product, there must be a minimum enzyme-substrate intermolecular distance, l_m at which binding interaction begins effectively. In order to determine l_m different values of the velocity (v) of hydrolysis of different mass concentration of the gelatinized potato starch with the same concentration of the enzyme, *Aspergillus oryzea* alpha amylase have to be determined. The V_{max} needs to be determined according to usual method [10]. Next, v should then be plotted versus the reciprocal of volume of solvent in which the substrate is dissolved or gelatinized. Since $v = k$ [S], v can be expressed as:

$$v = k\, m\, /V \tag{13}$$

where k and m are apparent rate constant and the mass of the substrate respectively. Thus $k\, m$ is the slope (S_L) of the plot of v versus $1/V$. $v_0V_o = S_L = v_1V_1$. Linearity in the plot is important for the determination of V at higher v and V_{max} in particular. The corresponding volume (V_{min}) at V_{max} is:

$$V_{min} = S_L/V_{max}\,. \tag{14}$$

Therefore,

$$l_m = (S_L/N_A V_{max}\,.\Sigma n)^{1/3} \tag{15}$$

Since $\Sigma n = n_s + n_2$, where n_s and n_2 are the number of moles of the substrate and the number of moles of the enzyme respectively.

The second aspect of this investigation concerns, the entropic term. This is not entropy of mixing or solution, but rather the effect of dilution and concentration which presents comparative aspect of the same issue of entropy; a diluted solution has higher "configurational entropy" (ΔS_{config}) than a concentrated solution. This seems to imply that the interparticle distance between the enzyme and substrate, l_x, for dilute solution should be longer than for concentrated solution. "Work" must be down to bring the particles closer within the region of their mutual interaction. That is there must be means of overcoming disorder. Two ways of increasing the concentration are: adding desirable amount of solid matter in the lowest entropic state into the solution producing two forms of entropy change viz: dispersion in solution resulting in TE and a decrease in ΔS_{config} for a single-solute solution containing either the substrate or the enzyme for instance. The mechanical aspect of weighing and adding to the solution is not related to entropy. The second approach is to concentrate by heating the dilute solution until the desired final volume is reached. If the volume lost largely at boiling point is ΔV then, the total entropy change is the sum of two aspects of entropy change, namely $\Delta V C_p \ln (T_b/T_0) /V_1$, (where V_1 is the molar volume of water ~ 18 exp (-6) m) the entropy change resulting from increase in temperature from initial temperature, $T_0 \rightarrow T_{nbp}$ (normal boiling point) and entropy of vaporization ΔVR $(4.5+\ln T_{nbp})/V_1$ in line with Trouton-Hildebrand-Everett rule. One may assume that C_p (molar heat capacity) is fairly constant against the backdrop of the status of being a solution rather than pure water. Increasing translational diffusion or rather translational velocity can enhance the rate of enzyme catalyzed reaction in highly diluted reaction mixture. This effect of increase occurs at higher absolute temperature. Another aspect of the translational velocity on account of implicit thermal energy is TE stated earlier.

A diluted reaction mixture unlike concentrated reaction has very high dilution entropy or rather configurational entropy (an entropic "barrier") the magnitude of which requires a great amount of work to overcome. This work is temperature dependent. Opposed to this barrier at any given temperature is TE. One can then postulate that the velocity of hydrolysis, v is α (TE-$\Delta V.\Delta S_{THERM}/V_1$) where $\Delta S_{THERM}(\{C_p \ln(T_{nbp}/T_0) + R$ $(4.5+\ln T_{nbp})\})$ is the total entropy change per mole of the solvent needed to eliminate excess solvent molecule so as to bring the reaction to a concentration that enables the minimum distance of closest approach between the enzyme and the substrate to be achieved:

$$v = \Theta\, [\text{TE}-\Delta V\{C_p \ln(T_{nbp}/T_0) + R\,(4.5+\ln T_{nbp})\}/V_1] \tag{16}$$

where Θ is proportionality constant. The work needed to concentrate the solution is reduced by the translational entropy which exists because the working temperature is much higher than absolute zero. $\Delta V.\Delta S_{THERM}/V_1$ is of quantitative value which merely enables the determination of the magnitude of entropic "barrier" constituted by the extent of dilution otherwise concentration by heating at 1 atmosphere can lead to denaturation of most enzymes, even though heating at much lower pressure can also lower the boiling point of water that can prevent denaturation. The concept of TE had been applied in the context of protein undergoing folding made possible by the

departing water of hydration, leading to gain in entropy opposed to conformational entropy [11]. The relevance lies in the translational diffusion/velocity of departing water of hydration of the macromolecule leading to TE gain. The deduction is that for any liquid or solution (reaction mixture) above freezing point of water there is always translational entropy. Therefore, a plot of v versus ΔV gives slope and intercepts as reflected in the Eq. (17) below.

$$v = \Theta TE - \Delta V\,\Theta\{C_p \ln(T_{nbp}/T_0) + R\,(4.5+\ln T_{nbp})\}/V_1 \quad (17)$$

From the slope (S_{LOPE}), Θ can be determined as follows:

$$\Theta = V_1 S_{LOPE}/\Delta S_{THERM} \quad (18)$$

From the intercept (I_{NT}), TE can be determined as follows:

$$TE = I_{NT}\Delta S_{THERM}\,/V_1 S_{LOPE} \quad (19)$$

2. MATERIALS AND METHODS

2.1 Materials

Aspergillus oryzea alpha amylase (EC 3.2.1.1) and soluble potato starch were purchased from Sigma – Aldrich, USA. Hydrochloric acid, sodium hydroxide, and sodium chloride, were purchased from BDH Chemical Ltd, Poole England. Tris 3, 5 – dinitrosalicylic acid, maltose, and sodium potassium tartrate tetrahydrate were purchased from Kem light laboratories Mumbia India, while potassium iodide was purchased from Merck Germany. Distilled water was purchased from local market.

2.2 Equipment

Electronic weighing machine was purchased from Wenser Weighing Scale Limited and 721/722 visible spectrophotometer was purchased from Spectrum Instruments China. *P*H meter was purchased from Hanna Instruments, Italy. Water bath was purchased from Hospibrand, USA.

2.3 Method

Stock solution of soluble potato starch was prepared by mixing 1 g in 100 ml of distilled water and subjected to heat treatment at 100℃ for 3 minutes, cooled to room temperature, and decrease in volume was corrected by topping the volume with distilled water to 100 mL to give 1.0 g%. Different concentrations of the substrate used in all assays were prepared by adding different volumes of distilled water, 7 ml, 6 ml, 5 ml, 4 ml, 3 ml, and 2 ml to 3 ml, 4 ml, 5 ml, 6 ml, 7 ml, and 8 ml of heat treated starch respectively. The molar mass of the potato starch is taken as 1000 kDa [12]. Stock solution of the enzyme was prepared by dissolving 0.01 g of the enzyme in 100 ml of tris – HCl buffer, whose *p*H is 6. Working stock concentration of the enzyme was prepared by subjecting the stock to 20-fold dilution to give 0.5 mg% (1.71 U/ml). The molar mass of the enzyme is taken as ~ 52 kDa [13, 14]. Each concentration of substrate is seen as one containing the same mass of potato starch in different volume of solvent. The concentration of the substrate which ranges from 3 g/l – 8 g/l is ≡ 10 g/3.333 l – 10 g/1.25 l. In order to determine the concentration at V_{max}, the minimum volume, V_{min}, need to be determined by plotting v versus reciprocals of different volumes-1/3.333 l, 1/2.5 l, 1/2 l, 1/1.67 l, 1/1.42857 l, and 1/1.25 l; V_{min} is then calculated according to Eq. (14).

Assay of the enzyme was carried out at 30℃ according to Bernfeld method [15]. Hydrolytic activity of the enzyme was terminated at three minutes by adding 1 ml of 3, 5 – dinitrosalicylic acid solution to 2 ml reaction mixture composed of 1 ml substrate and 1 ml enzyme. Spectrophotometric readings were taken at wave length = 540 nm. Molar absorption coefficient (ε) = 181.1 litre·mol^{-1}·cm^{-1} is determined according to usual Beer – Lambert equation: $A_{540} = \varepsilon\, C\, l$ where C and l are molar concentration of product and path length respectively while A_{540} is the absorbance. Kinetic parameters such as Michaelis – Menten constant (K_m) and V_{max} were determined according to Lineweaver – Burk method [10]. Other parameters were determined according to relevant equations specified in theoretical subsection.

2.4 Statistical Analysis

All values are expressed as mean ± SD. Microsoft Excel was used to calculate SD. Each parameter is an average of duplicate values.

3. RESULTS AND DISCUSSION

As Table 1 shows, the total ($t_{\infty x}$) and average (t_x) time spent outside the active site before complex

formation are expectedly decreasing with increasing [S]. This is so because the intermolecular distance between the enzyme and the substrate decreases with increasing [S]. The most important result is the observed increasing trend in total time (t_{Tc}) spent in the active site and the average time (t_c) (the time it takes to effect bond breaking and bond making, and product departure) with increasing concentration of the substrate. It seems instructive to accept the fact that greater time is needed in the active site for the transformation process than time outside the site, and that catalytic transformation is indeed not totally spontaneous. The duration of transformation in the active site is ≠ zero! Now the question is: how can macromolecular complex transform into biological entity spontaneously? This is in the light of the fact that high thermal energy may transform non spontaneous reaction to a spontaneous one but not without destabilizing effect on the expected biological entity. The kinetic parameters are shown under Table 1. The rate constant in particular was used to determine the interparticle distance, at which $t_c = 0$; the minimum solution volume which gives maximum [S] ($[S]_{max}$) at V_{max} and the minimum interparticle distance are shown under Fig. 1. This value of $[S]_{max}$ is similar to the K_m value reported earlier at 20℃ and *p*H, 5 [14]. However, the K_m in this research at 30℃ and *p*H is less than previous report. Nonetheless the scope of this research is limited to the use of generated data from assay to test the models. To be found under Fig. 2 are the interparticle distance (l_∞) at infinite dilution of the substrate, the volume (V_∞) of substrate solution at infinite dilution, and concentration ($[S]_{\infty dil}$) at infinite dilution. All these values for V_∞ and l_∞, are » corresponding values at $[S]_{max}$ which is » ($[S]_{\infty dil}$).

The proportionality constant, Θ (determined as illustrated in Fig. 3) for the determination of TE for reactant in the reaction mixture catalyzed by a fixed [E], the entropies of dilution at higher [S] and $[S]_{\infty dil}$, ΔS_{dil} and $\Delta S_{\infty dil}$ respectively, TE values at higher [S] and $[S]_{\infty dil}$ are shown under Table 1. Expectedly, the ΔS_{dil} values showed decreasing trend with increasing concentration of the substrate, with the highest reported at $[S]_{\infty dil}$. It can be observed that TE at infinite dilution is ≈ 217-fold larger than value reported for higher [S]. A very important deduction that needs to be made is that the TE value must be greater than ΔS_{dil} values and the entropic "barrier"; otherwise the activity of the enzyme may become less detectable or vanish entirely. At infinite dilution, $\Delta S_{\infty dil}$ is ≈ 2.24-fold larger than TE. But the value of $\Delta S_{\infty dil}$ is that which will require an amount of energy ($T.TE_\infty$) = 1,900.75 kJ/mol at 30^{o}C (being the temperature of assay) and atmospheric pressure, 760 mmHg to overcome it so as to enhance enzyme activity. Another point that needs to be made is that reactants in solution are not as mobile as gaseous reactant due to various factors. Longer time is therefore required in solution for reactive contact to be made despite the shorter interparticle distance in solution. There are reasons. "Though an electrostatic attractive or repulsive interaction between charged groups of a biomolecule or biomolecules is quite strong and long ranged in vacuum, it becomes about two orders of magnitude weaker and much shorter ranged in aqueous solution (~ 0.15 M-NaCl solution in biological systems) due to the screening effects" Kinoshita [16]. This makes the contribution of entropic factor to be very vital to the binding association of the enzyme and substrate or ligand bearing in mind that the entropic term may not necessarily be the kind based on the model of Kinoshita [16]. Experimental findings have shown that diffusion can be significantly hindered by electrostatic interactions between the diffusing particle and charged components of the extracellular matrix; the local diffusion coefficient decreases as the particle size increases or as the particle approaches the fibers [1]. This can also delay contact with the substrate.

Knowledge of the effective diffusion coefficients of reacting compounds is of crucial importance for the quantitative analysis of not just only bioprocesses using immobilized biocatalysts [17] but temporal analysis of enzyme catalyzed reaction. This is with a view to quantify the time expended at different stages of enzyme catalyzed reaction that can characterize different enzymes or homologous enzymes. Enzyme can be engineered to alter the functional groups in the active site or extra-active site locations which may either influence the site or alter the steric/bulk and hydration properties of the enzyme, all of which can either decrease or increase the translational diffusion coefficient of the protein. This should serve the need for either an increase or a decrease in the activity of any enzyme either for industrial or therapeutic application. Processes which can alter the mobility of enzymes in solution are amino acid substitutions or mutagenesis [18] which are useful as it has been shown that replacement of the active site His-15 of *Bacillus subtilis* HPr by negatively charged glutamate residue

Table 1. Duration of different aspects of *Aspergillus oryzea* alpha amylase catalytic action

[S]/g/l	Φ/exp (+4)	t_{Tc}/s	$t_{\infty x}$/s	t_x/exp (-5) s	t_c/ms	ΔS_{dil} /J/K
3	7.00±0.86	176.24±0.52	3.77±0.52	5.38±0.74	2.535±0.311	16.36±0.28
4	7.01±0.84	176.82±0.38	3.18±0.38	4.54±0.55	2.544±0.311	14.02±0.28
5	7.01±0.84	177.25±0.33	2.75±0.33	4.01±0.48	2.550±0.311	12.21±0.28
6	7.01±0.84	177.56±0.30	2.44±0.29	3.49±0.42	2.554±0.311	10.72±0.28
7	7.00±0.84	177.71±0.37	2.29±0.37	3.26±0.53	2.557±0.311	9.49±0.28
8	7.01±0.85	177.97±0.24	2.03±0.24	2.64±0.00	2.560±0.311	8.38±0.28
22.04±0.75	7.01±0.84	178.94±0.13	1.06±0.13	1.51±0.08	2.574±0.311	o<x<ΔS

Φ, t_{Tc}, $t_{\infty x}$, t_x, and t_c are number of catalytic cycle, total time spent within the active site, total time spent outside the active site or in the bulk, average time spent in the bulk, and average time spent in the active site during bond breaking and bond making, and product departure. The values of $(1/\langle k_2 \rangle) - \langle t_c \rangle$ range from 4.78-43.78 μs corresponding to value of [S] ranging from 22.04 g/l-3 g/l. $1/\langle k_2 \rangle$ = 2.58 ms. Results are approximated to 2 decimal places as may be applicable. "x" J/K implies that ΔS_{dil} may not be = 0 even if [S] → ∞. The corresponding activity ranges from 1.16±0.00-2.20±0.00 U/ml; Kinetic parameters are: K_m (8.79± 1.56 g/l); V_{max} (4.50±0.54 U/ml); k_2 (387.78±46.86/s): One unit is the amount of enzyme which gave 1μmol of reducing sugar, maltose, per min (Molar concentration (in μmol/l)× exp (-3)×V(in ml) /ml.min), exp (-3) is the conversion factor from ml to l. Θ = 3.98±0.10 exp(-4) mUmolK /Jml; TE = 28.95±0.03 J/mol.K. The entropy of dilution, $\Delta S_{\infty dil}$, at infinite dilution = 64.75±1.51 J/mol.K. V = 2 ml. TE (TE_∞) when t_c = 0, is = 6.27±1.13kJ/mol.K because v definitely → 0.

significantly reduced the overall conformational stability of the protein; but review report also shows that HPr is stabilized against urea induced unfolding following the substitution of Ala for His-15 [19]. Conformational entropy increase resulting to unfolding for function or dysfunction and conformational entropy decrease resulting to folding for stability for function will always lead to changes in hydration properties of the enzyme and consequently its mobility. Mobility and consequently TE cannot be extricated from conformational changes of a protein so long as the translational diffusion coefficient or velocity is affected.

Specific binding occurs through sites that must be properly aligned for the reaction to occur which has been described in a review by Kim and Yethiraj [5] as anisotropic reactivity as implied in the proposition that the enzyme diffuses towards the substrate and then lie side by side in order to interact and commence catalytic hydrolysis of the substrate [20]. This cannot be a timeless process. Time may be spent before appropriate alignment can be achieved with the possibility of being speeded up at higher concentration (Table 1) which reduces randomness or entropy (definitely not related to entropy of mixing) which is analogous to the increase in number density of gas molecules when it diffuses into smaller space. This is clearly illustrated in Table 1 where the time spent before contact with the enzyme is longer at lower [S]. Meanwhile the concern of this research is tied in part to translational entropy otherwise rotational diffusion translating to rotational entropy may also contribute to the magnitude of time it may take an enzyme to access appropriate orientation of the substrate for effective binding as implied in the anisotropic reactivity. This issue can be substantiated with the data in Table 1. But additional insight can be garnered with the impression $k_{turn} = 1/\langle T_{urn} \rangle$ [6] where k_{turn} (turnover rate) = k_{cat} [S]/([S]+K_m); This means that $1/k_{turn} > 1/k_{cat}$ and $\langle T_{urn} \rangle$ is average time it takes a single molecule of the enzyme to produce a single molecule of product as analyzed in theoretical subsection. This gives the impression that $\langle T_{urn} \rangle$ is longer for v than for V_{max} or for values of [S] < saturating [S]. But this research shows that the time expended in the active site is longer at higher [S] while the time outside the active before catalytic contact with the substrate is longer at lower [S] because longer interparticle distance is associated with lower [S]. Another valid deduction is that the values of t_c are time spent in the transformation of the substrate and departure of product; without departure of the product, the transformation or product formation is not complete. Thus $1/t_c$ is ≠ k_2(k_{cat}). Hence the time taken to yield a molecule of the product must not include the time taken for the enzyme to reach any more massive substrate within the period of assay. The control step is mainly the action in the active site which proceeds after appropriate catalytic orientation (which takes some time) has been achieved. Therefore, as may be observed in Table 1, $1/\langle k_2 \rangle$ (2.589 ms) is longer than any value of t_c. The values of $(1/\langle k_2 \rangle) - \langle t_c \rangle$ range from 4.78-43.78 μs corresponding to value of [S] ranging from 22.04 g/l-3 g/l. This seems to show that there is period of mutual catalytic orientation between the substrate and enzyme. Period of orientation can be affected by nature of the substrate or the physical state of the enzyme and

the extent of dilution. The state of dilution with its dilution entropy and excess intervening water molecules can inhibit the orientational process needed to bring the enzyme into catalytic state that entails conformational change. This is reflected in longer $(1/\langle k_2 \rangle) - \langle t_c \rangle$ at lower [S].

Even the most adapted cold-active enzyme cannot function if the reaction mixture is frozen or too cold, below optimal temperature for such enzyme for its function since there may be almost total loss of translational diffusion/velocity. At such condition the time spent in the active site should be zero even at infinite concentration of the reactants. Translational velocity or entropy is therefore essential for the generation of sufficient collision frequency between reactants; this is best served when TE is larger than entropy of dilution (and entropic barrier in particular defined in Eq. (16) as $\Delta V\{C_p \ln(T_{nbp}/T_0) + R(4.5+\ln T_{nbp})\}/V_1$) as shown in Table 1. The results and larger value of TE than ΔS_{dil} in this research confirm this position coupled with observed higher activity with lower dilution factor. There is no doubt therefore, that TE is very essential factor for enzyme function. This is supported in literature in which it is pointed out "that translational and (overall) rotational motion provide the important entropic driving force for enzymatic and intramolecular rate accelerations" [21].

The issue of TE and entropic "barrier" is not complete without consideration for the loss of TE upon substrate binding to the enzyme without which hydrolytic activity may remain elusive. Enzymes can carry out a large fraction of their extraordinary rate accelerations, by virtue of their ability to utilize substrate-binding forces to act as an "entropy trap" [21]. This presupposes that there should be loss of TE upon complex formation. Yet one needs to know why molecular associations in solution are opposed by the loss of entropy that results from the restriction of motion of each of the components in the complex [4]. However, the claim that "low-energy conformational changes that are possible within the enzyme-substrate complex are disadvantageous with respect to the contribution of this entropy effect (TE loss) to the catalytic process" seem to call to question the known fact that cold adapted enzymes are in their highest possible conformational flexibility or entropy so as to function at low temperatures [22]. "The enzyme's functional properties are intimately, governed by protein motions and conformational changes" [22]. Conformational changes have specific interpretation unlike protein motion which may mean internal motion or its time-dependent translational displacement which is the concern of this research. This duration of displacement is shown in Table 1.

Since no catalytic cycle is complete without product release, it is important to consider partial refolding under ambient condition of the enzyme. This is where TE of the solvent molecule becomes applicable as explained by Harano and Kinoshita [11]. Partial refolding implies that the enzyme retains some conformational dynamics or flexibility in preparation for interaction with other fragment or full-length substrate molecule under ambient temperature. As pointed out in submitted manuscript (I.I. Udema), any mesophilic enzyme for instance, in solution at room temperature, may assume partial conformational expansion leading to increased entropy as modeled by Fitter [9].

This research has shown to a very large extent that no reaction can be extremely spontaneous as not to require a life-span. Hence time must be expended in the active site, and the magnitude of such time is characteristic property of the enzyme. Besides it is not understatement to posit on the basis of this result that the extent of dilution merely prolongs the time it will take to transform a given mass of reactant to product; reaction may be impossible at infinite dilution because the entropic barrier, and not necessarily the entropy of dilution $\rightarrow\infty$. The following discussion can elucidate the claim. Two different [S]s say, 10 g/100 ml and 15 g/100 ml, can give different amount of product when subjected to the action of an enzyme for the same duration under the same condition. When the two concentrations are subjected to 2-fold dilution, the mass of the dissolved substrate remains the same, the concentration is halved, and the entropy of dilution for both concentrations is $R\ln 2$/J/mol.K. Thus it is the entropic "barrier" for any given [S] that need to be surmounted by TE in order to enhance catalytic function. At a given temperature or increasing temperature, both TE and conformational entropy will increase leading to increase in activity for a given [S]. This is supported by the observation that enzyme under study undergoes two discrete transitions at 30 and 45℃ to attain a more dynamic conformational substate [23].

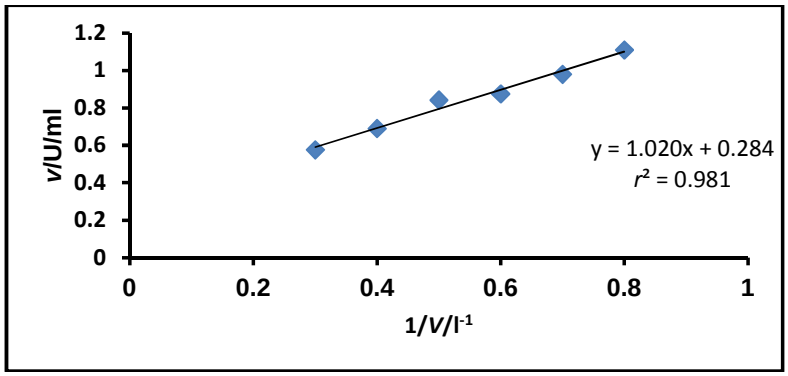

Fig. 1. Determination of the regression coefficient (slope) for the calculation of the minimum volume (V_{min}) of substrate solution at maximum velocity, V_{max}, of hydrolysis of substrate

V is volume of solvent in which the same mass of substrate is mixed to give any desired concentration. V_{min} = 0.454±0.016l; l_m=5.3142±0.06 exp(-8) m; Maximum [S]([S]$_{max}$)= 22.04±04 g/l

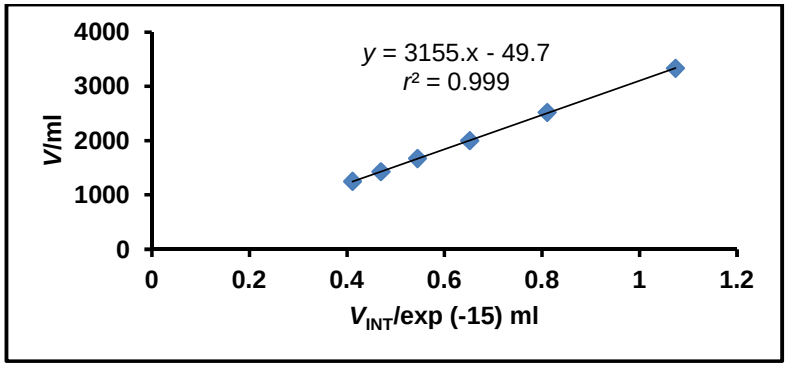

Fig. 2. The regression of volume (V) of the substrate solution on average interparticle volume (V_{INT}) for the determination of volume (V_∞) at infinite dilution of the substrate concentration

V is volume of solvent in which the same mass of substrate is mixed to give any desired concentration; Total reaction mixture volume = 2 ml. As shown in Eq. (12), l_∞ = 7.03±0.04 exp (-7) m; V_∞ = 1.103±0.199 exp(6) ml. Prepared [S] for assay ranges from 3 g/l – 8 g/l ≡ 10 g/3.333 l – 10 g/1.25 l (see Table 1 for detail); the concentration $[S]_{\infty dil}$ at "infinite dilution" 9.215±1.659exp(-3) g/l ≡ 10 g/1103.49 l

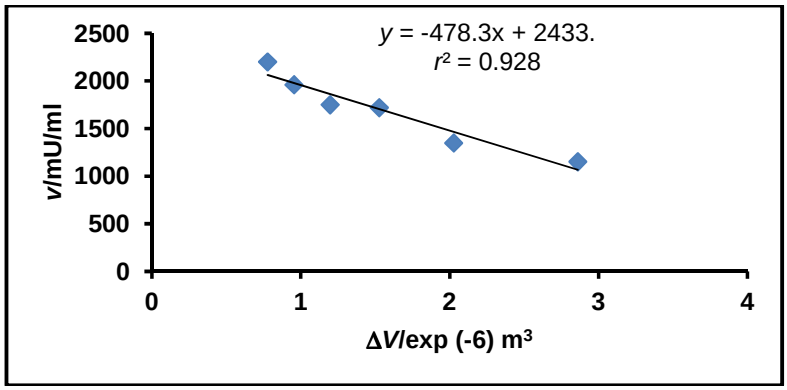

Fig. 3. The regression of v on excess volume (ΔV) for the determination of translational entropy (TE) according to Eqs. 17, 18 and 19

4. CONCLUSION

Enzyme catalyzed reaction is both concentration and time dependent process promoted and also hindered by entropic factors. The time outside the active site before effective collision of the enzyme with the substrate is shorter than the time spent within the site. There is time spent in the course of catalytic orientation of the enzyme and substrate for effective binding; thus the time spent in the active site for transformation of substrate and release of product is shorter than the time it takes a molecule of enzyme to yield a product. Catalytic action of the enzyme is opposed by entropic "barrier" due to excess solvent implicit in the extent of dilution, but enhanced by TE whose value for the enzyme investigated in this study is 28.95±0.03 J/mol.K.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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