Title: A Two-part Approach to the Determination of Intrinsic Rate Constants of an Alpha-amylase Catalysed Reaction.

ABSTRACT

Background: There is a need for equations with which to calculate the intrinsic rate constants that can further characterise enzyme catalysed reactions despite what seems to be conventional differences in methodology in the literature.

Methods: Theoretical, experimental (Bernfeld method), and computational methods.

Objectives: 1) To derive an alternative intrinsic rate constants equations consistent with their dimension, 2) derive electrostatic intermolecular potential energy equation, **(ξ_e)**, 3) calculate the intrinsic rate constants for forward (k_1) and reverse (k_2) reactions, and 4) define the dependence or otherwise of kinetic constants on diffusion and deduce the catalytic efficiency.

Results and discussion: The ultimate quantitative results were ~ 64.69 ± 0.49 exp (+3)/ min (k_2) (and k_d (σ) = ~ 60.66 exp (+3)/ min) , ~ 1594.48 ± 11.99 exp (+3) L/mol.min (k_1) (and k_a (σ) = ~1482.47 exp (+3) L/mol.min), ~ 58.00 ± 10.83 exp (+3) /min, the apparent rate constant for reverse reaction (k_b), and ~ 75.83 ± 10.83 exp (+3) /min, the rate constant for product formation (k_3). The catalytic efficiency was: 3.025 exp (+ 9) L / mol.

Conclusion: The relevant equations were derived. Based on the derived equations the intrinsic rate constants can be calculated. Since k_3 is > k_b, the k_3 is diffusion controlled and it appears that the enzyme has reached kinetic perfection. The evaluation of rate constants either from the perspective of diffusion dependency or independency cannot be valid without Avogadro number.

Keywords: *Aspergillus oryzea* alpha-amylase, apparent rate constants, intrinsic rate constants, intermolecular electrostatic potential energy, diffusion control, kinetic perfection.

1. INTRODUCTION

Researchers have shown interest in what has been termed diffusion controlled and non-diffusion controlled enzyme catalysed reactions [1-3]. The confusion that may arise is that there could not be an encounter complex formation without any form of motion. Coupled to this is the concept of intrinsic rate constants [1, 2] whose equations need to be redetermined. Solute molecules which are immobile in their various positions cannot form an encounter complex let alone solute-solute (*e.g.* enzyme-substrate) complex. However, with reference to the works of Allison and McCammon [4] and Elcock *et al*. [5], Lu and McCammon [6] posit that electrostatically steered diffusion-reaction processes exist widely in chemistry and biochemistry. Similar to this is the view that "to bind at an enzyme's active site, a ligand must diffuse or be transported to the enzyme's surface, and, if the binding site is buried, the ligand must diffuse through the protein to reach it [3].

Although the driving force for ligand binding is often ascribed to the hydrophobic effect, electrostatic interactions, also, influence the binding process of both charged and nonpolar ligands [3]. Although Wade *et al* [3] claimed that electrostatic steering is particularly relevant for diffusion controlled reaction, it needs to be made clear that binding (or preferential interaction by binding) cannot occur without ultimate attractive electrostatic interaction. In line with this view is the claim that "as time increases, the rate coefficient decreases because the enzymes must diffuse to the substrate in order for reaction to occur" [7] following binding which brings motion close to zero.

Besides, it has been observed that for an enzyme in solution, the rate-determining step in catalysis will be either k_f, the rate of *ES* formation, or simply k_3, the rate of product formation [8]. If k_3 is rate limiting, the catalytic events that occur after substrate binding are slower than the rate of the formation of the *ES* complex [8]. If, however, k_f is rate limiting, the enzyme turns over essentially instantaneously once the *ES* complex has formed [8]. In either case, according to Copeman [8], the fastest rate of catalysis for an enzyme in solution is limited by the rate of diffusion of molecules in the solution. This statement seems to imply that regardless of the rate limiting step, there must be initial translational motion (this may exclude randomness within certain intermolecular distance).

Many years ago, in the 20th century, the concept of intrinsic rate constants was advanced. Recently, the paper by Eser and Fitzpatrick [9], "Measurement of intrinsic rate constants of tyrosine

hydroxylase reaction", showed greater clarity for the effect of viscogens on catalytic efficiency than was the case for intrinsic rate constant. Since rates and rate constants in particular are important characteristics of enzyme catalysed reactions for industrial applications, therapeutics (activation and detoxification of drugs), digestion, a step to the end of nutrition *etc*, there is a need to rederive or restate the equation for the determination of such intrinsic rate constants with consistency in the units of the kinetic constants. This is against the backdrop of the observation in the literature to the effect that, in Shurr's [1] equation, if generalisability is possible, the parameter $\rho_{eq}(r)$ designated as g, is exp $(+U(r)/k_BT)$ unlike Vijaykumar *et al* [10] who defined $\rho_{eq}(r)$, given as exp $(-U(r)/k_BT)$, as the equilibrium probability that they (the substate and the enzyme for instance) are at the distance, r from each other. One may wish to know if this observation is on the basis of conceptual differences. "It could not be ideological differences". What is ultimately important is the need for equations with which to compute the intrinsic rate constants that can further characterise enzyme catalysed reactions despite what seems to be conventional difference in methodology in the literature which indicates a possibility of a two-part approach for the determination of intrinsic rate constants. Nevertheless, redefinition of K_D and its application and, the adoption of Vijaykumar *et al* [10] approach are to be addressed in the appendix section. Thus, the objectives of this research are: 1) To derive an alternative intrinsic rate constants equations consistent with their dimension, 2) derive electrostatic intermolecular potential equation, 3) calculate based on derived equations the intrinsic rate constants, and 4) define the dependence or otherwise of kinetic constants on diffusion.

2.0 Theory

In this investigation one begins with the premise that if the solute-solute attraction is greater than solute-solvent attraction, the aqueous solute particles may coalesce and precipitate out of solution; this may be applicable to a dilute solution which becomes concentrated due to substantial partial evaporation. It may arise due to continuous addition of the soluble solute. This is applicable to all solutes, the ionic and nonionic solutes. Indeed any solvent, be it either polar organic – or nonpolar organic – solvent in which a solute is dissolved is applicable. This is also against the backdrop of the claim that the intermolecular potential ($U(r)$) is concentration-dependent] [1]. In this research however, the literature material of immense interest and relevant is the work by Shurr [2].

2.1 Diffusion – dependent and diffusion – independent rates

According to the Shurr [2] “the reactions for which the rate constant for product formation and release, k_3 is not diffusion-dependent include almost all reactions whose equilibria lie far to the side of the products, since the rate of association of enzyme, *E* and products, *P* to form enzyme-substrate complex, *ES* will generally be sufficiently slow in these cases that it is not diffusion dependent, and consequently the conjugate dissociation step k_3 will not be diffusion dependent either”. “Only extremely rapid over-all reactions have the possibility of a diffusion-dependent k_3 when the equilibrium greatly favors the products, and such cases cannot be experimentally characterised by the usual steady-state methods in any case”. This view seems to go against the earlier view because far right position of equilibria implies that the product formation is more favoured and, as such, k_3 in the scheme below may be » k_b.

$$E + S \underset{k_b}{\overset{k_f}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P \qquad (1)$$

Where, k_f and k_b are the rate constant for the forward reaction, rate constant for the dissociation of *ES* to free enzyme, *E* and free substrate, *S*.

Based on the assumption that k_3 is not diffusion controlled, the Michaelis – Menten constant, $K_M = (k_b + k_3)/k_f$ is the only part of the expression which depends upon the diffusion coefficient. “Clearly, for saturation (i.e. $[S_0]$ » K_M) conditions, K_M may be neglected and diffusion plays no role in the reaction, however fast” [1]. Also, if k_3 « k_b, then $K_M \cong k_f/k_b = k_2/k_1$ (where k_2 and k_1 are the intrinsic rate constants for the backward reaction, $ES \rightarrow E + S$ and for the formation of *ES* respectively) so that the K_M and also velocity of catalytic action, Φ are independent of diffusion processes [1]. Going by these statements, despite what seems to be a contradictory statement at the beginning of the paragraph, it seems that the K_M and Φ are independent of diffusion only when k_3 « k_b. Also, if k_3 is » k_b, k_3 should be diffusion dependent. Finally, if the k_f and k_b are not appreciably diffusion controlled (i.e. $f\,k_l$ « $g\,k_D$), then K_M and, hence, Φ will not be diffusion dependent [1]. The concern in this statement is that while *g* and *f* to be given shortly, are dimensionless, k_1 and k_D (to be given shortly) are neither dimensionless nor of the same dimension. The factor, *g* is given as

$$g = \exp\left(U(r)/k_B T\right) \quad (2)$$

Where, $U(r)$, k_B and T are the intermolecular potential energy, Boltzmann constant and thermodynamic temperature respectively.

Meanwhile,

$$f = R\int_R^{\infty} \exp\left(U(r)/k_B T\right)\frac{\mathrm{dr}}{r^2} \quad (3)$$

Where R (= R_E + R_S) is the reaction radius where R_E and R_S are the hydrodynamic radii of the enzyme and substrate respectively.

$$k_D = 4\,\pi(R_E + R_s)(D_E + D_s) \quad (4)$$

The unit of k_D is m^3/s because the diffusion coefficients for the enzyme, D_E and substrate, D_S is m^2/s and the unit of R_E or R_S is the metre; the unit of k_1 is 1/M.min. Therefore, $f\,k_l$ and $g\,k_D$ cannot be compared qualitatively.

For a diffusion-dependent reaction in the absence of forces

$$K_M = \frac{k_2}{k_1} + \frac{k_3}{k_f} = K_{eq}^{-1} + \frac{k_3}{k_D}\left(\frac{k_D + k_1}{k_1}\right) \quad (5)$$

However, if it is known that $k_3 \gg k_b$, then if $k_3/k_D \approx K_M$ (which may not be case because the unit of k_3 is 1/min and that of k_D is m^3/s), it may be concluded that the reaction is diffusion dependent. While the forces in question are not specified in the original work [1], nevertheless the equation $K_M = \frac{k_2}{k_1} + \frac{k_3}{k_f}$ may be likely, unlike the equation, $K_M = K_{eq}^{-1} + \frac{k_3}{k_D}\left(\frac{k_D + k_1}{k_1}\right)$ because k_1 and k_D do not possess the same unit. However, it may be speculated that such force may be intermolecular potential force referred to in paper one [1]. Besides, k_2 and k_3 are 1st order rate constants (mol/mol/unit time) while k_1 and k_f are 2nd order rate constants ((dm^3/mol)/unit time).

Summarising, Shurr [1] posits that the usual enzyme reaction is independent of diffusion and, hence, the medium viscosity under the following circumstances: (a) $[S_0] \gg K_M$ and (b) $k_3 \ll k_b$; the enzyme reaction is diffusion-dependent under the following circumstances: (a) $[S_0] \lesssim K_M$ and (b) $k_3 \gg k_b$. Based on this summary, one can evaluate the experimental data generated under the given conditions so as to determine any dependence or otherwise on diffusion.

In the first paper by Shurr [2] the equations of k_b, the effective steady-state reverse rate constant and k_f, the effective steady-state forward rate constant were stated without any derivational process. The equations are given as

$$k_b = \frac{k_D k_2 \exp(U(r)/k_B T)}{f k_1 + g k_D} \quad (6)$$

$$k_f = \frac{k_D k_1}{f k_1 + g k_D} \quad (7)$$

Once again, it is necessary to restate that the concept of intrinsic rate constants may be quantitatively useful in biochemical, medical, and biological sciences in general. Intrinsicality may be an inherent tendency for a physicochemical process to occur. This may be applicable to biochemical reactions leading to one or more effects such as enhanced rate constants in general if factors that can enable the process exist. Thus, while Eq. (6) and Eq. (7) contain the intrinsic rate constants, the presence of only a single k_D in the denominator in both equations renders them dimensionally invalid. As in the literature [2], the equations may be restated with k_D appearing twice in the denominator by replacing the k_1 in Eq. (6) with K_D leading to the following results.

$$k_b = \frac{k_2 \exp(U(r)/k_B T)}{f + g} \quad (8)$$

Looking critically at Eq. (8) one sees that the nominator contains *g* such that *f* + *g* as denominator should be > *g*; this implies that the k_b is a fraction of the 'k_2'. Applying the same procedure to Eq. (7) gives

$$k_f = \frac{k_1}{f + g} \quad (9)$$

Before further comment, it needs to be stated that *g* may be constant for a given system. Thus, making the denominator in Eq. (6), Eq. (7), Eq. (8), and Eq. (9) subject of the formula and upon rearrangement one obtains

$$g = \frac{k_1 k_b}{k_2 k_f} \quad (10)$$

Meanwhile for a reaction to occur the reactants must be within reach of each other as applicable to binding interaction between the substrate and enzyme. The substrate molecules are randomly distributed (Fig. 1). The introduction of an aliquot of enzyme solution starts a reaction after translational motion, the effect of swirling notwithstanding. Figure 2 illustrates the intermolecular distance in which randomness is negligible as the molecules approach each other. This is where diffusion becomes

universally relevant the distinction between diffusion – dependent and diffusion – independent catalytic action of enzymes notwithstanding.

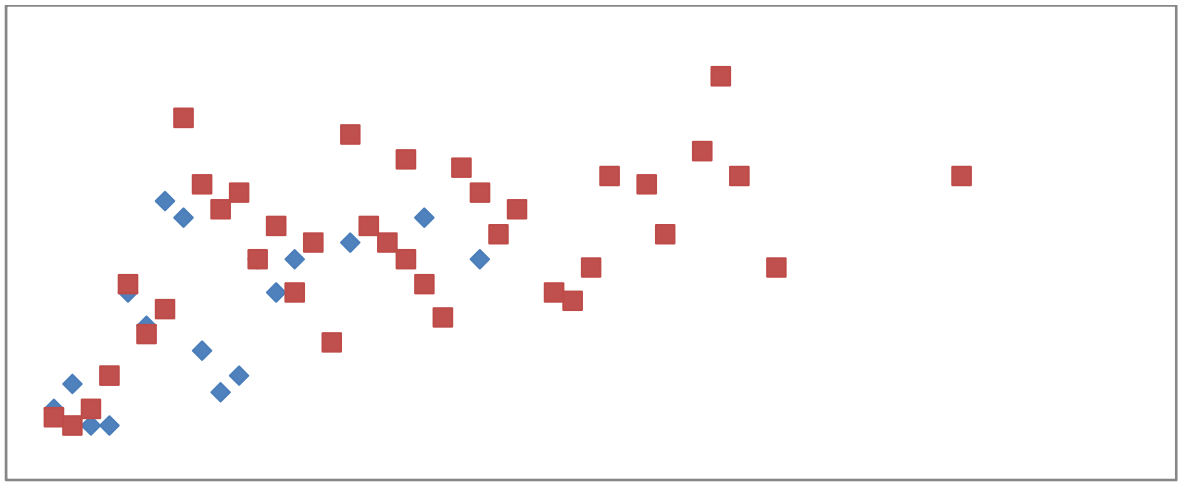

Fig.1: Reaction mixture containing the molecules of enzyme and substrate. *Blue (◊) and red (□) stand for the enzyme and the substrate molecules respectively. The positions of the symbols depict randomness. The substrate molecules are previously in random distribution before the addition of the aliquot of the enzyme solution, though swirling rapidly distributes the molecules of enzyme.*

E _____ *S*

Fig. 2: Initial intermolecular distance (R_x): *Initial intermolecular distance where the there is infinitesimal tendency for randomness; the velocity of the solute is bulk-like.*

If one is not mistaking, Eq. (3) seems to suggest that two particles are to be separated from an intermolecular distance equal to *R* to an infinite intermolecular distance; however in this research the coming together of the enzyme and substrate is also of interest because there is a need to be aware of the electrostatic force of attraction between the enzyme and the substrate. The equation for this is given in method subsection. Since the reaction radii can be seen to be constant, there must be a distant apart from which mutual weak electrostatic perturbation of the combining molecules occurs (Fig. 3); attractive perturbative interaction cannot occur at an infinite intermolecular distance if consideration is given to conservative forces.

→ *E* —— *S* ←

Fig. 3: Change in intermolecular distance with time
The linear interval —— illustrates the minimum intermolecular distance (R_0) needed for the commencement of electrostatic attraction. The longer arrow portrays the fact that the smaller molecule, the enzyme made a longer displacement than the substrate.

The outcome of Eq. (3) (whose physical meaning was not stated in the original work [2]) based on attractive interaction can be stated as

$$f = Rg\left(\frac{1}{r_1} - \frac{1}{r_2}\right) \tag{11}$$

Where, $r_2 > r_1$ and $r_1 = R$; $r_2 > R$. A careful examination of Eq. (11) should reveal that f is < 1 but > 0. Although R has been defined, but in this research, it is replaced with the intermolecular distance (R_{ter}) where terminal velocity is attained. On the basis of this and the fact that g is always > 1 if the intermolecular potential energy is negative, then k_b and k_f are fractions of k_2 and k_1 respectively. Next is the determination of the alternative equation of g based on what has been described as diffusion – dependent and diffusion – independent kinetic constants.

2.2 Determination of alternative equation of the intermolecular potential energy

The intermolecular potential energy for the diffusion-independent case depends on the relationship given as [1].

$$\frac{K_M}{M_{alt}} = \frac{k_2}{k_1} \tag{12}$$

Making k_2 subject of the formula and substitute same into Eq. (10) gives

$$g = \frac{k_b M_{alt}}{k_f K_M} \tag{13}$$

It is clear here that g must always be > 0 and, it could be >1 but « ∞ if U (r) is negative.

Therefore,

$$U(r) = k_B T \ln\left(M_{alt}\, k_b / K_M k_f\right) \tag{14}$$

The intermolecular potential energy for the diffusion-dependent case requires the following equation [1]. However, this may appear to be a contradiction considering the fact that Eq. (15) below refers to a case in the absence of forces that are not explicitly defined. Clearly, subsequent derivation may confirm this absence of forces. Thus,

$$\frac{K_M}{M_{alt}} = \frac{k_2}{k_1} + \frac{k_3}{k_f} \tag{15}$$

The reason for the appearance of the molar mass of maltose, the product of amylolysis has been explained elsewhere [11]. Making k_2 subject of the formula and substituting into Eq. (10) gives:

$$g = \frac{k_b}{(K_M/M_{alt} - k_3/k_f)k_f} \quad (16)$$

Therefore,

$$U(r) = k_B T \ln \frac{k_b}{(K_M/M_{alt} - k_3/k_f)k_f} \quad (17)$$

However, $k_b = (K_M/M_{alt} - k_3/k_f)k_f$ confirming the fact that $U(r) = 0$. Additional reason is advanced in method section. It is however, difficult to concede to the notion of zero intermolecular force if not mistaken for something else. But the implication is that, the intermolecular distance approaches infinity if not infinite, going by the concept of conservative field forces. Biochemical transformation is unlikely at infinite dilution. The values of the k_b and k_f can be determined by fitting the equations in literature [11] to the data generated experimentally. The equations are given in the method subsection.

2.3 Considering electrostatic kinetic energy as a key factor in the catalytic function of enzyme.

While it is obvious that potential and kinetic energies are convertible, the interest in the latter is due to its direct link with translational motion which ensures delivery to target. Attraction begins when minimum intermolecular distance is reached. There should be an initial increase in velocity, a decrease due to viscosity, a steady velocity and sudden decrease to ≈ zero velocity (Fig. 4).

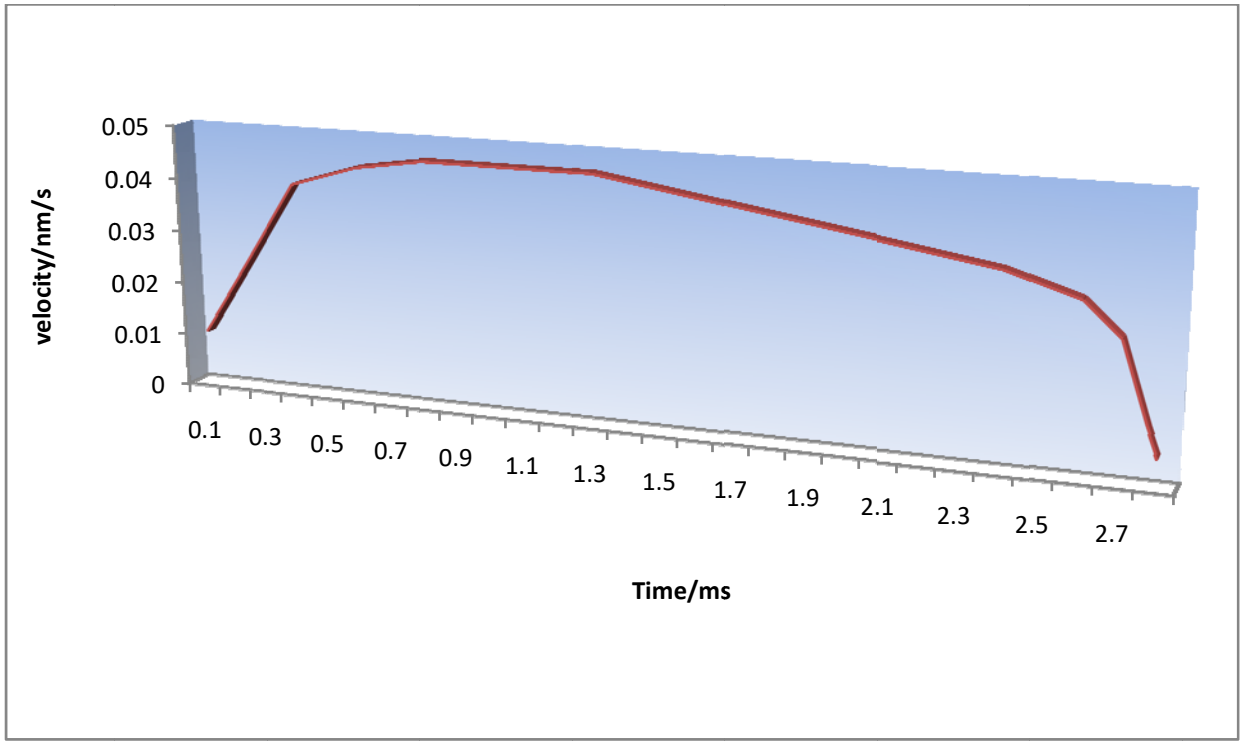

Fig. 4. Hypothetical time course of the velocity changes as solute molecules approach each other attractively: *At the commencement of attractive interaction there is an increase in velocity, followed by a decrease to velocity > than bulk, and finally to ≈ zero velocity as complex formation occurs. The decrease is due to solvent resistance otherwise called viscosity. The initial bulk-like and final (which* $\rightarrow 0$*) velocities are more important and relevant than the increase in velocity. Once again the blue background symbolises the fact that the reaction occurred in aqueous medium.*

In a previous investigation [12], the need for minimum intermolecular distance for the commencement of attractive electrostatic interaction was established. This is relevant to very dilute reaction mixture of the enzyme and substrate in laboratory test tubes unlike *in vivo* cases where the concentration of pancreatic [13] plus intestinal alpha-amylase [14-15] is known to be very high. It is known that "substrate concentrations within cells are in the neighbourhood of their K_M values (exp (– 6) – exp (– 2) M); with reference to Cha [15], Goldstein [16] and Srere [17], Schnell and Maini [14] posit that this scenario enhances the full potential of the enzymes or the intrinsic capacity of the enzyme to executes its function as may be expressible via the intrinsic reverse and forward rate constants [2]. The 1st step in this regard is to derive electrostatic interaction energy otherwise called interaction potential equation. The derivation is based on the assumption that the total work down in transit between a position in bulk before collision and after collision is equal to the sum of the work down within the electrostatic field and outside the field. Thus,

$$F_{\mathrm{Tot}}(\mathfrak{R}-R)=\frac{k_{\mathrm{B}}T}{L}(\mathfrak{R}-R_{\mathrm{ter}})+F_{\mathrm{Elect}}(R_{\mathrm{ter}}-R) \tag{18a}$$

Where, $\mathfrak{R}$, F_{Tot}, L, F_{Elect} and R_{ter} are the concentration-dependent bulk intermolecular distance where U(r) $\rightarrow 0$, total force, the cube root of the molar volume of water, the electrostatic force of attraction, and the intermolecular distance where terminal velocity is attained. F_{Tot} is given as in manuscript in preparation as:

$$F_{\mathrm{Tot}}=\frac{\left(\frac{\mathfrak{r}(\mathfrak{R}-R)}{m}+\sqrt[2]{\left(\frac{\mathfrak{r}(\mathfrak{R}-R)}{m}\right)^2+\left(\frac{Lu_\theta^2}{k_{\mathrm{B}}T}\right)^2}\right)(k_{\mathrm{B}}T)^2}{(u_\theta L)^2} \tag{18b}$$

$\mathfrak{r}$ = 0.97471916 [12] and u_θ is determined as described in the literature [18] and manuscript in preparation; $u_\theta=\sqrt[2]{\frac{\sqrt[3]{4m(k_{\mathrm{B}}T\,D_{\mathrm{E}}/L)^2}}{m}}$ where D_{E} and m are respectively, the diffusion coefficient and mass of the enzyme molecule. The electrostatic force is given as:

$$F_{\text{Elect}} = \frac{F_{\text{Tot}}(\Re - R) - \frac{k_B T}{L}(\Re - R_{\text{ter}})}{R_{\text{ter}} - R} \quad (18c)$$

The electrostatic energy (ξ_{Elect}) is given as:

$$\xi_{\text{Elect}} = F_{\text{Elect}} R_0 \quad (18d)$$

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

Aspergillus oryzea alpha-amylase (EC 3.2.1.1) and soluble potato starch were purchased from Sigma – Aldrich, USA. Tris 3, 5 – dinitrosalicylic acid, maltose, and sodium potassium tartrate tetrahydrate were purchased from Kem light laboratories Mumbai, India. Hydrochloric acid, sodium hydroxide, and sodium chloride were purchased from BDH Chemical Ltd, Poole England. Distilled water was purchased from local market. The molar mass of the enzyme is ~ 52 k Da [19].

3.1.2 Equipment

Electronic weighing machine was purchased from Wenser Weighing Scale Limited and 721/722 visible spectrophotometer was purchased from Spectrum Instruments, China; pH meter was purchased from Hanna Instruments, Italy.

3.2 Methods

3.2.1 Preparation of solution of reactants and assay.

The enzyme was assayed according to Bernfeld method [20] using gelatinised potato starch whose concentration range is 4-10 g/L; the weight average molecular weight of the insoluble potato starch is 7.73 exp (+7) g/mol [21]. Reducing sugar produced upon hydrolysis of the substrate using maltose as standard was determined at 540 nm with extinction coefficient equal to ~ 181 L/mol.cm. Concentration equal to 1 g/100 mL of potato starch was gelatinised at 100 ^{o}C for 3 min and subjected to serial dilution after making up for the loss of moisture due to evaporation to give concentrations ranging between 4 and10 g/L. Concentration equal to 0.1 g/100 mL of *Aspergillus oryzea* alpha-amylase was prepared by dissolving 0.1 g of the enzyme (as the stock) in 100 mL of Tris HCl buffer at pH = 6.9. Assay of the enzyme was carried out with an enzyme concentration = 1 mg/L. The duration of assay was 3 minutes at 25 ^{o}C.

3.2.2 The determination of kinetic constants

Alternative direct linear plot [22] was explored for the determination of K_M, the Michaelis – Menten constant and v_{max}, the maximum velocity of amylolysis. The equations used for the determination of kinetic constants are [11, 23]:

$$k = M_{alt}\left(\frac{v_{max} \pm \sqrt[2]{v_{max}^2 - 4\,S_{lope}[S_0]}}{2[S_0]}\right) \quad (19)$$

Where, v_{max}, s_{lope}, $[S_0]$, k, and M_{alt} are the maximum velocity of amylolysis, slope from the plot of velocity of amylolysis, v versus $[S_0]/(v_{max} - v)$, concentration of the substrate, first order rate constant for the utilisation of the substrate, S and molar mass of maltose as product respectively.

$$\mathrm{I\,n}\frac{1}{1-\frac{[E_0]M_{alt}}{[S_0]}\mathrm{I\,n}\frac{[E_0]}{[E_0]-[ES]}} = k\,t \quad (20)$$

Where, $[E_0]$, t, and $[ES]$, are the molar concentration of the enzyme at t = 0, duration of ES formation and molar concentration of enzyme-substrate complex.

$$\mathrm{I\,n}\frac{[E_0]}{[E_0]-[ES]} = \frac{(k_{-1}+k_2)[S_0]}{K_M k}(1-\exp(-k\,t)) \quad (21)$$

However, without prejudice to Eq. (20) and the graphical approach in literature [11], it has been realised that $\mathrm{I\,n}\frac{1}{1-\frac{[E_0]M_{alt}}{[S_0]}\mathrm{I\,n}\frac{[E_0]}{[E_0]-[ES]}} = (1-\exp(-k\,t))$ if the product of the calculated values of t (Eq. (20)) and calculated values of k based on Eq. (19) is substituted into Eq. (21). This can be interpreted to mean that $\mathrm{I\,n}\frac{[E_0]}{[E_0]-[ES]}$ can be plotted against $[S_0]\left(\mathrm{I\,n}\frac{1}{1-\frac{[E_0]M_{alt}}{[S_0]}\mathrm{I\,n}\frac{[E_0]}{[E_0]-[ES]}}\right)/k$ to yield a slope = $\frac{(k_{-1}+k_2)}{K_M}$ from where, $k_{-1} + k_2$ is given as slope × K_M. Ultimately, the k_f is then given as: slope × M_{alt}.

3.2.3 The determination of intermolecular distance for electrostatic attraction.

The determination of the minimum intermolecular distance for the commencement of electrostatic attraction (being also the beginning of negative potential energy of interaction in line with conservative field force principle) is as previously described. The equation is given below.

$$R_0 = R/\left(1-\left(S_{lope}(1)/\left(S_{lope}(2)\right)^2\right)\right) \quad (22a)$$

Where R (which is = R_E + R_S where R_E and R_S are taken as the radii of spheres whose diffusion coefficients are equal to that of the species, the enzyme and substrate respectively being considered by

exploring Einstein-Stoke equation); $S_{lope}(1)$ is the 1st slope from the plot of the square of effective collision frequency (ν) versus $1/\mathfrak{R}$ ($\mathfrak{R} - R$) and $S_{lope}(2)$ is the 2nd slope from the plot of ν versus $1/\mathfrak{R}$ where $\mathfrak{R}$ is the concentration-dependent intermolecular distance. The frequency of collision, $\nu \approx 2\pi RDC_E$ where C_E ([*ES*] N_A) is expressed in number of molecules per cubic metres, where N_A is the Avogadro number), and D is taken to be equal to the sum of the D_S and D_E. The D_S for potato starch was calculated using the relationship: $D_S = D_v\sqrt[3]{(M_v/M_S)}$ where D_v, M_S and M_v are the diffusion coefficient (which is 1.31 exp (–11) m^2/s) of tomato bushy virus [24] at 298.15 K, weight average molecular mass of potato starch, and molar mass of virus given as 1.06 exp (+7)/mol [24].

In order to determine the dimensionless factor f at intermolecular distance where terminal velocity is reached, such intermolecular distance (R_{ter}) needs to be determined. Hence, as in the manuscript in preparation, the R_{ter} can be calculated with Eq. (22b) below.

$$(R_{ter} - R)^2\left((24\pi^2\eta R_E\,\mathfrak{r}_2 R\,D_{E\to\infty}C_E(u_\theta L)^2)^2 - \frac{48\pi^2\eta R_E\mathfrak{r}_2^2 R\,D_{E\to\infty}C_E(u_\theta L)^2(k_B T)^2}{m_2}\right) = (Lu_\theta^2 k_B T)^2 \quad (22b)$$

Where η, R_E, C_E = [*ES*]N_A (where [*ES*] is in mol/m^3 and N_A is the Avogadro number), $\mathfrak{r}$ = 0.97471916 a factor which enables the calculation of distance covered by the smaller particle of two particles moving towards each other as described elsewhere [12] and $D_{E\to\infty}$ is given as in the same manuscript under preparation as:

$$D_{E\to\infty} = \frac{\left(\frac{48\,\pi^2\eta\,R_E\mathfrak{r}_2^2 R\,C_E(u_\theta L)^2(k_B\theta)^2}{m_2} + \sqrt{\left(\frac{48\,\pi^2\,\eta\,R_E\mathfrak{r}_2^2\,R\,C_E(u_\theta L)^2(k_B\theta)^2}{m_2}\right)^2 + 4\left(24\,\pi^2\eta R_E\mathfrak{r}_2 R\,C_E(u_\theta L)^2\right)^2\frac{(Lu_\theta^2 k_B\theta)^2}{(\mathfrak{R}-R)^2}}\right)}{2\left(24\,\pi^2\eta\,R_E\,\mathfrak{r}_2 R\,C_E(u_\theta L)^2\right)^2} \quad (22c)$$

3.2.4 The generalisable equations for the determination of intrinsic rate constants.

Having determined the equation for ξ_e, the dimensionless factor g, given as Eq. (2) can be determined such that the 2nd dimensionless factor f can also be determined given the value of R_0. The method for the determination of the latter is given as Eq. (22a). One can obtain the reverse intrinsic rate constant by rearranging Eq. (8) to give

$$k_2 = k_b(f + g)/g \quad (23)$$

Likewise the forward intrinsic rate constant is obtained by rearranging Eq. (9) to give

$$k_1 = k_f(f + g) \quad (24)$$

Having previously defined f as $g(1-R/R_0)$, Eqs (23) and (24) is restated respectively as:

$$k_2 = k_b\,(2 - R/R_0)\ (\text{Thus } k_2 \neq \boldsymbol{f}\,(g)) \quad (25)$$

$$k_1 = k_f\,g(2 - R/R_0)\ (\text{Thus } k_1 = \boldsymbol{f}\,(g)) \quad (26)$$

Equations (25) and (26) can be applied in a straight forward manner in the determination of relevant intrinsic rate constant.

3.3 Statistical Analysis

The standard deviation was determined according to the method described by Hozo *et al* [25] and by means Microsoft Excel. The mean values of 3 determinations were used to determine all the effective kinetic constants.

4. RESULTS AND DISCUSSION.

This research clearly is not concerned with rate constants mainly but there is a need to state equations of intrinsic rate constants that are dimensionally consistent as to be very much applicable to biochemical and even biophysical processes that need quantification. There is also important need to characterise enzyme catalysed reactions as either diffusion – dependent or diffusion – independent reaction. In this regard, there is always a need to bear in mind that, be it diffusion – dependent or diffusion – independent reaction, there is always initial intermolecular motion due to attractive interaction and thermal energy. Hence there is the diffusion-limited rate constant, k_D which determines the rate at which the two particles (*e.g*. enzyme and substrate) diffuse towards each other [26].

The parameter k_D is adopted for the determination of what may the termed apparent (or effective) rate constants, the 2nd order rate constant, k_f for *ES* formation and the 1st order rate constants, k_b for the dissociation of *ES*. In this regard, Vijaykumar *et al* [10] derived k_f and k_b in line with what they called Agmon and Szabo [26] procedure to give equations (which are different from Eq. (6) and Eq. (7) [1]) where $\rho_{eq}(r)$ (i.e. $\exp(-U(r)/k_BT)$) was defined as the equilibrium probability that they are at the distance, r from each other. In Shurr's [1] equation, if generalisability is possible, the parameter $\rho_{eq}(r)$ designated as g, is $\exp(+U(r)/k_BT)$. One may wish to know if this observation is on the basis of conceptual differences. "It could not be either conventional or ideological differences". Despite these commendable efforts, the issue of dimension remains unresolved. This issue was intuitively resolved as shown in Eq. (8) and Eq.

(9). Based in part on the method in literature [11] it is possible to calculate the intermolecular potential which enabled the calculation of the equilibrium probability [26], a necessary requirement for the calculation of intrinsic rate constants (Table 1). Though not shown in any table, it needs to be revealed that substitution of relevant data into Eq. (14) gave ~ – 2.07 kJ/mol as interaction potential for a diffusion-independent reaction or rate constant if that was the case; for the diffusion-dependent reaction or rate constant, $U(r) = 0$. This implies that no *ES* may have been formed.

Table 1. Apparent and intrinsic rate constants

Results obtained based in part on modified Shurr's approach			
k_3 /min	**k_b /min**	**k_f/L/mol.min**	***g***
75.83 ± 10.83 exp (+3)	58.00 ± 10.83 exp (+3)	1417.48 ± 0.20 exp (+3)	
([*S*]+[*E*])/mol/L/exp (–8)	**k_2/ min/exp (+3)**	**k_1 /L/mol.min**	
7.098	63.83	1617.32	0.910
8.391	64.42	1596.00	0.900
9.685	65.13	1584.25	0.891
10.979	64.77	1593.19	0.896
12.272	65.05	1586.26	0.892
14.860	64.91	1589.86	0.894
Average ± SD	64.69 ± 0.49	1594.48± 11.99	0.897 ± 0.007
Results obtained based in part on modified Vijaykumar *et al* approach			
$k_{off}(\sigma)$	~ 60.66 exp (+3) /min		
$k_a(\sigma)$	~ 1482.47 exp (+3) /min		

The rate constants, k_f, k_b, k_3, k_2 ($k_{off}(\sigma)$), and k_1($k_a(\sigma)$) are the 2nd order rate constant for enzyme-substrate formation (ES), reverse rate constant for the dissociation of ES, rate constant for product formation, and the intrinsic rate constants, the dissociation rate constant for the formation of E (enzyme) and S (substrate) and the association rate constant for the formation of ES respectively. Total enzyme concentration is ~1.923 exp (– 8) mol/mL; [S] + [E] and g values are approximation to 3 decimal places while the rest are approximations to 2 decimal places. The Michaelis-Menten constant and maximum velocity of amylolysis are 32.29 ± 6.04 g/L and 1458.34 ± 208.35 μM/min; the catalytic efficiency is: 3.025 exp (+9) L / mol. In line with Shurr's [1] approach, $U(r) = k_BT$ In g.

Based on another approach in this research, Eq. (23) to be specific, the attractive energy per molecule was ~ 1.02 exp (– 21) J but need not be used further. Taking in part, the approach of Shurr [2], the values of $\rho_{eq}(r)$ (or *g* which is = exp ($+U(r)/k_BT$)) were determined as shown in Table 1. Having known

the value of a dimensionless parameter, f (Eq. (11)) - calculated after replacing r_2 with R_0) - and g, the intrinsic rate constants were calculated according to Eq. (25) (and Eq.A.15b) and Eq. (26) (and Eq. A. 18b). The modified approach of Vijaykumar *et al* [10] yielded values that are similar (though the magnitudes differ) to any of the results from modified approach of Shurr [14]. Unlike modified Shurr's approach, modified approach of Vijaykumar *et al* did not require information about g (or $\rho_{eq}(r)$) for the computation of the intrinsic rate constants. The calculated values were > the apparent rate constants as shown in Table (I). These results seemed to suggest that enzymes can achieve higher rates if challenge of viscosity and greater stability of the enzyme can be attained. As shown in Table (1), the apparent rate constant for product formation is > the reverse rate constant for the dissociation of *ES* to free enzyme and substrate. This means that the substrate undergoes conversion to product as quickly as the *ES* is formed [8]. The rate limiting step is thus, the formation of *ES*. Cognate to this is the issue of catalytic perfection [8] which requires the catalytic efficiency to be very high as in this research as shown as footnote under Table 1.

5. CONCLUSION

The equations for the calculation of intrinsic rate constants and were re-stated with dimension consistent with the kinetic parameters determined. The equation for intermolecular electrostatic potential energy is exactly derivable. The intrinsic rate constants could be higher than the apparent rates constants. The apparent rate constant (k_3) for product formation and release is > the apparent reverse rate constant for the release of free enzyme, E and free substrate, S. Thus, the k_3 may be diffusion controlled. With reservation it seems the enzyme has attained kinetic perfection under the assay condition. Besides, the research has shown that certain parameters cannot be validly quantified, without Avogadro number.

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APPENDIX

Further redefinition of intrinsic rate constants

In this section Vijaykumar *et al* approach is introduced so as to enable the creation of results that may compare with the results obtained using Shurr's approach. Meanwhile taken k_D given as $k_D = 4\pi(R_E + R_s)(D_E + D_s)$ leaves one with a dimensional issue that has been addressed in the main text. But its application has been tied however, to the caveat that, when intermolecular distance is chosen to be beyond the range r_c (intermolecular distance allowing for mutual electrostatic interaction) of the interaction potential, then an exact expression given above for K_D, the Smoluchowski diffusion-limited reaction rate constant is applied [10]. However, one need to know of what value this could be if in line with

conservative field principle, the potential energy of interaction is zero as to imply that no electrostatic attraction of the enzyme for the substrate occurs. The application is reflected in Eq. (A.3) and Eq. (A.4) for intrinsic association rate constant and intrinsic dissociation rate constant respectively. Meanwhile in line with Vijaykumar *et al* [10] the corresponding equations expected if Shurr's approach is taken into account are:

$$k_{\mathrm{b}} = \frac{k_2 \exp(-U(r)/k_{\mathrm{B}}T)}{f + \exp(-U(r)/k_{\mathrm{B}}T)} \tag{A.1}$$

$$k_{\mathrm{f}} = \frac{k_1}{f + \exp(-U(r)/k_{\mathrm{B}}T)} \tag{A.2}$$

Where, in line with Vijaykumar *et al* approach [10], g (or $\rho_{eq}(r)$) is given as exp ($-U(r)/k_{\mathrm{B}}T$).

$$k_{\mathrm{on}} = \frac{k_{\mathrm{a}}(\sigma) k_{\mathrm{D}}(\sigma)}{k_{\mathrm{a}}(\sigma) + k_{\mathrm{D}}(\sigma)} \tag{A.3}$$

Again the unit of association rate constant (k_{on}) and its corresponding intrinsic rate constant (k_a (σ)) is dm^3/mol per unit time, while $k_D(\sigma)$ is strictly dm^3 per unit time. The same dimensional issue is applicable to Eq. (A.4) below because the unit of first order rate constants, apparent or effective rate constant and the corresponding intrinsic rate constant is a dimensionless quantity per unit time. The mole concept must not be precluded!

$$k_{\mathrm{off}} = \frac{k_{\mathrm{d}}(\sigma) k_{\mathrm{D}}(\sigma)}{k_{\mathrm{a}}(\sigma) + k_{\mathrm{D}}(\sigma)} \tag{A.4}$$

Where k_{off} and $k_d(\sigma)$, the effective dissociation rate constant and the corresponding intrinsic dissociation rate constant are in dimensionless quantity per unit time. Making $k_a(\sigma) = k_D(\sigma)$ leads to k_{on} being = $k_a(\sigma)/2$ and k_{off} being = $k_d(\sigma)/2$. These are definitely different from Eq. (24) and Eq. (23) respectively. This is despite the fact that the intrinsic values remain > than the effective rate constants. The bone of contention is therefore, the concern for validity. However, in the literature [27] is the equation given as:

$$k_D = 4\,\pi\, N_A (R_E + R_s)(D_E + D_s) \tag{A.5}$$

Where, N_A is the Avogadro constant.

Making k_a (σ) and k_d (σ) in Eq. (3) and Eq. (4) respectively subject of the formula gives respectively

$$k_{\mathrm{a}}(\sigma) = \frac{k_{\mathrm{on}} k_{\mathrm{D}}(\sigma)}{k_{\mathrm{D}}(\sigma) - k_{\mathrm{on}}} \tag{A.6}$$

$$k_{\mathrm{d}}(\sigma) = \frac{k_{\mathrm{off}}(k_{\mathrm{a}}(\sigma)+k_{\mathrm{D}}(\sigma))}{k_{\mathrm{D}}(\sigma)} \quad \text{(A.7)}$$

In order that Eq. (A.6) to be valid, $k_{\mathrm{D}}(\sigma)$ must be > k_{on}. To be noted is the fact that application of $4\pi(R_{\mathrm{E}} + R_{\mathrm{s}})(D_{\mathrm{E}} + D_{\mathrm{s}})$ as the K_{D} makes it of no consequence because it is « 1. This could be seen clearly if Vijaykumar *et al* approach (Eq. (A.6) and Eq. (A.7)) is critically examined. Such a scenario in addition to the issue of dimension calls to question the approaches of Vijaykumar *et al* [10] and Shurr [1]. However, the substitution of $4\pi N_{\mathrm{A}}(R_{\mathrm{E}} + R_{\mathrm{s}})(D_{\mathrm{E}} + D_{\mathrm{s}})$ in place of k_{D} should give a dimensionally and scientifically more consistent result. It is the introduction of Avogadro number that gives a correctional effect. This is thus, effected beginning from Eq. (A.6) as follows:

$$k_{\mathrm{a}}(\sigma) = \frac{4\pi k_{\mathrm{on}}(R_{\mathrm{E}} + R_{\mathrm{S}})(D_{\mathrm{E}} + D_{\mathrm{S}})N_{\mathrm{A}}}{4\pi(R_{\mathrm{E}} + R_{\mathrm{S}})(D_{\mathrm{E}} + D_{\mathrm{S}})N_{\mathrm{A}} - k_{\mathrm{on}}} \quad \text{(A.8)}$$

The function (σ) is dropped in order to avoid technical confusion. Since $k_{\mathrm{a}}(\sigma)$ is never practicably a negative parameter, $4\pi(R_{\mathrm{E}} + R_{\mathrm{s}})(D_{\mathrm{E}} + D_{\mathrm{s}})N_{\mathrm{A}}$ must always be > k_{on} which may not be the case without N_{A}. Moreover, $\frac{4\pi(R_{\mathrm{E}} + R_{\mathrm{S}})(D_{\mathrm{E}} + D_{\mathrm{S}})N_{\mathrm{A}}}{4\pi(R_{\mathrm{E}} + R_{\mathrm{S}})(D_{\mathrm{E}} + D_{\mathrm{S}})N_{\mathrm{A}} - k_{\mathrm{on}}} > 1$ so that $k_{\mathrm{a}}(\sigma)$ is always greater than k_{on}.

Subjecting Eq. (A.7) to similar treatment gives:

$$k_{\mathrm{d}}(\sigma) = \frac{k_{\mathrm{off}}(k_{\mathrm{a}}(\sigma)+4\pi(R_{\mathrm{E}} + R_{\mathrm{S}})(D_{\mathrm{E}} + D_{\mathrm{S}})N_{\mathrm{A}})}{4\pi(R_{\mathrm{E}} + R_{\mathrm{S}})(D_{\mathrm{E}} + D_{\mathrm{S}})N_{\mathrm{A}}} \quad \text{(A.9)}$$

Once again, the intrinsic dissociation constant, a 1st order rate constant, is always > k_{off} because $\frac{(k_{\mathrm{a}}(\sigma)+4\pi(R_{\mathrm{E}} + R_{\mathrm{S}})(D_{\mathrm{E}} + D_{\mathrm{S}})N_{\mathrm{A}})}{4\pi(R_{\mathrm{E}} + R_{\mathrm{S}})(D_{\mathrm{E}} + D_{\mathrm{S}})N_{\mathrm{A}}} > 1$.Though this claim is mathematically valid for both Eqs. (A.8) and (A.9), the issue of potential energy of interaction being zero as the maximum value, in line with conservative field principle, remains relevant because, $U(r)$ is zero at infinite dilution as to imply that enzyme-substrate interaction may be nonexistence.

In order that binding can take place there must be a form of attractive interaction between the bullet and target molecule such as enzyme and substrate, drug and deadly pathogen *etc*; this presupposes that there should be kinetic energy and consequently negative potential of interaction, a key characteristics of conservative field principle. As applied to equations arising from Shurrs approach, the equation of the equilibrium probability that two molecules are at the distance, *r* from each other is derived as follows:

$$k_{\mathrm{a}}(\sigma) + k_{\mathrm{D}}(\sigma) = \frac{k_{\mathrm{a}}(\sigma)\rho_{eq}(\sigma)k_{\mathrm{D}}(\sigma)}{k_{\mathrm{on}}} = \frac{k_{\mathrm{d}}(\sigma)k_{\mathrm{D}}(\sigma)}{k_{\mathrm{off}}} \quad \text{(A.10)}$$

Simplification and rearrangement of Eq. (A.10) gives as follows an equation exactly the same as that derived from Shurr's given equations:

$$\rho_{eq}(\sigma) = \frac{k_{\mathrm{d}}(\sigma)k_{\mathrm{on}}}{k_{\mathrm{a}}(\sigma)k_{\mathrm{off}}} \quad \text{(A.11)}$$

The equations, $k_a = k_a(\sigma)$, $k_d = k_d(\sigma)$ and $k_D = k_D(\sigma)$ means that these rate constants, in contrast to the effective rate constants k_{on} and k_{off}, depend on the choice of σ [Vijaykumar *et al*]. This simply means that any of the intrinsic rate constants is a function of 'σ'.

Meanwhile, the scientist, the biochemist in particular in the subfield, enzymology, professionals such as medics, pharmacists, dieticians and nutritionist is interested on the fate of food or drug as the case may be; this may preclude the value of σ that is beyond the range r_c of the interaction potential, where $U(r)$ may be equal to zero. In such situation, binding of the enzyme to food substrate or drug as may be applicable and the drug to the pathogen may be impossible due to over dilution. Thus a negative $U(r)$ (or equivalently the kinetic energy) is desirable. This implies that the equilibrium probability $\rho_{eq}(\sigma)$ (Eq. (A.11)) needs to be reintroduced. Thus,

$$k_{\mathrm{on}} = \frac{k_{\mathrm{a}}(\sigma)\rho_{eq}(\sigma)k_{\mathrm{D}}(\sigma)}{k_{\mathrm{a}}(\sigma)\rho_{eq}(\sigma)+k_{\mathrm{D}}(\sigma)} \quad \text{(A.12)}$$

$$k_{\mathrm{off}} = \frac{k_{\mathrm{d}}(\sigma)k_{\mathrm{D}}(\sigma)}{k_{\mathrm{a}}(\sigma)\rho_{\mathrm{eq}}(\sigma)+k_{\mathrm{D}}(\sigma)} \quad \text{(A.13)}$$

From Eq. (A.12),

$$k_{\mathrm{a}}(\sigma) = \frac{k_{\mathrm{on}}k_{\mathrm{D}}(\sigma)}{(k_{\mathrm{D}}(\sigma)-k_{\mathrm{on}})\rho_{\mathrm{eq}}(\sigma)} \quad \text{(A.14)}$$

Upon substitution of Eq. (A.11) into Eq. (A.14) and simplification one obtains,

$$k_{\mathrm{d}}(\sigma) = \frac{k_{\mathrm{D}}(\sigma)k_{\mathrm{off}}}{k_{\mathrm{D}}(\sigma)-k_{\mathrm{on}}} \quad \text{(A.15a)}$$

In order not to slip into former confusion, $k_D(\sigma)$ as $4\pi(R_E + R_s)(D_E + D_s)N_A$ is substituted into Eq. (A.15a) to give:

$$k_{\mathrm{d}}(\sigma) = \frac{4\pi(R_{\mathrm{E}} + R_{\mathrm{S}})(D_{\mathrm{E}} + D_{\mathrm{S}})N_{\mathrm{A}}k_{\mathrm{off}}}{4\pi(R_{\mathrm{E}} + R_{\mathrm{S}})(D_{\mathrm{E}} + D_{\mathrm{S}})N_{\mathrm{A}}-k_{\mathrm{on}}} \quad \text{(A.15b)}$$

From Eq. (A.13), $k_d(\sigma)$ is also given as:

$$k_{\mathrm{d}}(\sigma) = \frac{k_{\mathrm{off}}(\sigma)\left(k_{\mathrm{a}}(\sigma)\rho_{\mathrm{eq}}(\sigma)+k_{\mathrm{D}}(\sigma)\right)}{k_{\mathrm{D}}(\sigma)} \quad \text{(A.16)}$$

Substitution of Eq. (A.11) into Eq. (A.16) gives respectively 1st after rearrangement and 2nd after making $(k_a^2)(\sigma)$ subject of resulting equation the following:

$$\frac{k_{\mathrm{off}}^2 k_a^2}{k_{\mathrm{d}} k_{on}} = \frac{k_{\mathrm{D}}^2 k_{\mathrm{off}}}{k_{\mathrm{D}} - k_{\mathrm{on}}} - k_{\mathrm{off}} k_{\mathrm{D}} \quad \text{(A17a)}$$

$$k_{\mathrm{a}}(\sigma) = \sqrt[2]{\left(\frac{k_{\mathrm{D}}(\sigma)}{k_{\mathrm{D}}(\sigma) - k_{\mathrm{on}}} - 1\right)\frac{k_{\mathrm{d}}(\sigma) k_{\mathrm{on}} k_{\mathrm{D}}(\sigma)}{k_{\mathrm{off}}}} \quad \text{(A.17b)}$$

Simplification of Eq. (A.17 b) gives finally

$$k_{\mathrm{a}}(\sigma) = \frac{k_{\mathrm{on}} k_{\mathrm{D}}(\sigma)}{k_{\mathrm{D}}(\sigma) - k_{\mathrm{on}}} \quad \text{(A.18a)}$$

$$k_{\mathrm{a}}(\sigma) = \frac{4\pi(R_{\mathrm{E}} + R_{\mathrm{S}})(D_{\mathrm{E}} + D_{\mathrm{S}}) N_{\mathrm{A}} k_{\mathrm{on}}}{4\pi(R_{\mathrm{E}} + R_{\mathrm{S}})(D_{\mathrm{E}} + D_{\mathrm{S}}) N_{\mathrm{A}} - k_{\mathrm{on}}} \quad \text{(A.18b)}$$

Equations (A.15a)/(15b) and (A.18a)/(A.18b) have the same denominator and most importantly as usual, all the independent variables (or parameters) can either be theoretically (in particular with respect to k_D (σ)) or experimentally with respect to k_{on} and k_{off} determined. While it is obvious that binding interaction is a function of attractive kinetic energy which must diminish in favour of increasing potential energy during dissociation, the determination of intrinsic rate constants does not require information about the potential energy of interaction for their determination as long as the background approach of Vijaykumar is the case. One should not shy away from the fact that the equilibrium probability is equal to one if potential energy of interaction is substantially negative (or substantial and sustained mutual electrostatic attraction yielding kinetic energy) as to engender enzyme-substrate formation for instance, leading to catalysis of whatever kind. Substitution of Eqs (A.15b) and (A.18b) into Eq. (A.11) verifies this view.

With respect to Vijaykumar *et al* [10], Eqs (8) and (9) may imply that where *U(r)* is equal to zero, on account of σ being > r_c, a case of infinite dilution, there can never be any form of association or encounter complex formation preceding enzyme-substrate complex formation, and, if there has never been association there could never be any dissociation. On the other hand with respect to Shurr [2], Eqs (25) and (26) show respectively that where $R_0 \rightarrow \infty$, $k_b \approx k_2 / 2$ and $k_1 = 0$ because $g = 0$ ($U(r) = 0$ at infinite dilution). However, there is no question of $k_b \approx k_2 / 2$ because if there was no association, there can never be dissociation of *ES*.

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