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Non-equilibrium Binding Energy Determined Using Alpha-amylase Catalysed Amylolysis of Gelatinised Starch as a Probable Generalisable Model and Importance

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Author's contribution

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

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ABSTRACT

Objectives: This research was undertaken to determine the non–equilibrium binding energy by calculation after substituting experimental data into derived equations, present its role distinct from energy associated with activated enzyme–substrate (*ES*) complex and ultimately elucidate the importance of binding energies.
Background: There are overwhelming pieces of evidence in the literature that binding interaction is essential for the ultimate transformation of a substrate, inhibition of vital enzymes of pathogens, covid-19 in particular. Intrinsic binding energy herein referred to as non–equilibrium binding energy and energy associated with activated *ES* are seen to be chemical in origin. Much attention seemed not to be given to theoretical approach to the determination of non–equilibrium binding energy.
Methods: Experimental approach (Bernfeld method of enzyme assay) and calculational.
Results and Discussion: The non–equilibrium translational (2.691–2.726 kJ/mol) and total electrostatic energies (2.755–3.154 kJ/mol) were > than the thermal energy at 310.15 k. The interfacial distance between the bullet and target molecule was expectedly very short; the range was between 6.672 and 7.570 exp (– 12) m. This was attributed to the interaction between charged enzyme and weakly polar substrate.

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Conclusion: The equations of non–equilibrium and translational energies were derivable. The binding interaction serves to fix the bullet molecule on or into the target (supra) molecule before the commencement of transition state formation. The non–equilibrium binding interactions of the bullet (drugs, substrate, *etc*) and target (receptors *e.g.* enzymes, pathogens such as Covid–19, *Plasmodium etc*) and the ultimate complex are likely to be stabilised against the thermal energy in furtherance of enzymatic and drug action since the electrostatic interaction energy is higher than thermal energy.

Keywords: Gelatinised insoluble potato starch; human salivary alpha–amylase (EC 3.2.1.1); non–equilibrium interaction energy; total translational energy; total electrostatic interaction energy.

1. INTRODUCTION

There is no reason for now to suggest that substrate transformational process such as hydrolysis, modification *etc* can occur without enzyme–substrate complex formation after intermolecular interaction and physically controlled binding. This is regardless of the mechanism of enzyme action. There are models for explaining how an enzyme binds to a substrate [1]. Early understanding of the mechanism of enzyme action (or function) is anchored on the "induced fit" hypothesis or model of Daniel Koshland jr [2] and "lock and key hypothesis" of Emil Fischer [3]. Both models seem to suggest ways by which ultimate enzyme substrate binding can occur. With reference to the literature [4–6], Pan [1] observed that the flexibility of the active site [4], the spatial adaptation [5] and structural plasticity of proteins *i.e.* the enzymes to be specific, have been experimentally verified; yet, despite past debate about which of the hypothesis or model should be adopted, the induced fit model seems to be gaining upper hand with the advent of "conformation selection" principle [7–11] which postulates that all of the potential conformations of a given protein preexist and that once the ligand selects the most favored conformation, induced fit occurs and conformational change takes place [1]. All these notwithstanding, one should realise that nature has psychrophilic (Psy), mesophilic (Mes), and extremophilic (Ext) organism whose enzymes, alpha–amylase for instance, function under different temperature conditions; this is to imply that the enzymes' active sites exist in different degrees of structural/conformational flexibility in the following order Psy > Mes > Ext [12]. This enables function at different temperatures. This temperature differences can be illustrated as follows: « 273.15 k for the Psy enzymes; » 273.15 k but « 323.15 k for the Mes enzymes and » 323.15 k but < 500 k for the Ext enzymes [12]. The normal body temperature of humans is known to be 310.15 k.

Research has shown that "stronger and longer–ranged interaction between ligand and protein favours the induced–fit model, and weaker and shorter-ranged interaction leads to the population–shift model' [8]. The authors "further postulate that the protein binding to a small ligand tends to proceed via the population-shift model [8], whereas the protein docking to macromolecules such as DNA tends to fit the induced-fit model" [1]. There has also been the view that the population–shift model was proved to be applicable to the antigen–antibody binding interaction and substrate binding to the enzyme [1]. However, there is a need to opine that charged enzyme and substrate at a given normal pH may exhibit a long-range interaction unlike where uncharged substrate like starch and similar macromolecular substrate is the case.

Thus, despite the assertion that substrate unbinding [13] promotes faster rate of enzymatic action in recent past, and regardless of the mechanism, "induced fit and luck and key", there are overwhelming pieces of evidence in the literature that binding interaction and cognate binding energy are essential for the ultimate transformation of substrate, inhibition of vital enzymes of pathogens, Covid-19 in particular *etc* [14–16]. Much attention seemed not to be given to experimentally amenable theoretical approach to the determination of intrinsic binding energy (renamed as non–equilibrium binding energy in this research) as distinct from Gibbs free binding energy. Be it either "induced-fit or luck and key", there must always be binding interaction whose enabling force and energy in particular needs to be properly defined in terms of either state or path function. The intrinsic binding energy is defined as the total interaction energy between

the substrate molecules and enzymes' functional groups [17]. Incidentally, the Gibbs free energy of binding of substrate to the enzyme (or vice versa) mainly is reported in the literature [14–16] for most enzymes other than amylases. There is however, less information on what has been termed intrinsic binding energies [18] with surprising interpretation by some authors [19]. This research is thus, undertaken to determine the non–equilibrium binding energy by calculation after substituting experimental data into derived equations, present its role distinct from energy associated with activated enzyme–substrate (*ES*) complex, and ultimately elucidate the importance of binding energies using human salivary alpha amylase and insoluble gelatinised starch as model.

2. REVIEW OF THEORY

Previous research has shown that the effective energy of any dissolved molecule in the solution may not be exactly $k_B\,\theta$ because of both solvent resistance and cohesive force. Recently the principle of friccohesity has been developed and applied [20], but cannot be applied here. Both forces need not be measured as they remain the major factors that influence the mobility of solution components. However, the derived equation depended on Einstein–Stoke equation where viscosity constant is very relevant. The equation [21] which has undergone modification [22] for the calculation of instantaneous velocity of soluble solute in solution is given as:

$$\xi_2 = \sqrt[3]{4m_2\left(\frac{k_B\,\theta\,D_\theta}{L}\right)^2} \qquad (1)$$

Where, ξ_2, m_2, k_B, θ, D_θ, and L are the effective thermal energy of the protein (enzyme), mass of a molecule of the enzyme, Boltzmann constant, thermodynamic temperature, translational diffusion coefficient, and the cube root of the molar volume (in cubic metres) of water respectively. Division of Eq. (1) by the mass of a molecule of the enzyme and taking the square root gives the velocity as soon as the solute dissolves and subsequently decreases to lower velocity as terminal velocity due to solvent viscosity. The instantaneous velocity is however, used for the determination of parameters stated in method section and explained in the appendix section. This velocity according to Eq. (1) is expectedly much less than root mean square velocity expected of molecules in gaseous state otherwise it would imply, "in a reverse evolutionary process" that biological fluid can speed–up as in gas phase with tragic consequences (total dehydration) and death.

This research showcases the possibility of quantifying the total translational energy (thermal energy + energy originating from electrostatic attraction) which results in higher translational velocity without the influence of aqueous solvent medium with the understanding that such parameter should be quantitatively different from bulk solution value just as the root mean square velocity is much greater than the instantaneous effective velocity during the dissolution of a solute as explained earlier. Thus, taking ξ_{trans}*(dry)* as the total translational energy without the effect of aqueous solvent medium (the cohesive force being applicable during dissolution), $k_B\theta$ and ξ_2 in Eq. (1) need to be replaced to give:

$$\xi_{eff}\,(total) = \sqrt[3]{4\,m_2\left(\frac{\xi_{\mathrm{trans}}\,(dry)\,D_\theta}{L}\right)^2} \qquad (2)$$

Where, ξ_{eff} *(total)* is the total effective translational energy (thermal energy + energy originating from electrostatic attraction), and, it is given as:

$$\xi_{eff}\,(total) = m_2\left(\left(\frac{\mathcal{F}_{\mathrm{elect}}}{6\,\pi\,\eta\,R_E}\right)^2 + u_\theta^2\right) \qquad (3)$$

Where, $\mathcal{F}_{elect}$ (Eq. (A.34)) is the total electrostatic force of attraction derived in the appendix section and u_θ^2 the square of the instantaneous translational velocity is given as ξ_2 (Eq. (1)) /m_2; η is the viscosity coefficient equal to 0.697 kg/m s at 310.15 k [23] and R_E is the hydrodynamic radius of the enzyme. Substitution of Eq. (3) into Eq. (2), rearrangement, and making ξ_{trans} *(dry)* subject of the formula gives:

$$\xi_{\mathrm{trans}}\,(dry) = \frac{m_2\,L}{2\,D_\theta}\left(\left(\left(\frac{\mathcal{F}_{\mathrm{elect}}}{6\,\pi\,\eta\,R_E}\right)^2 + u_\theta^2\right)^3\right)^{½} \qquad (4)$$

The total electrostatic energy of attraction (Eq. (A.35)) is given as $\xi_{(LS)} = \mathcal{F}_{elect}\,R_0$ where R_0, the intermolecular distance at the beginning of electrostatic attraction is determined as described in method section.

In this research classical mechanics, translational velocity, (de) accelerative forces and kinetic energy of motion, attraction or potential energy of interaction and enzyme kinetics are applied rigorously. This is with the understanding that interaction between

macromolecules could be attractive, repulsive, and neutral depending on the intermolecular distance. It is on account of this that previous related research [24] was reviewed and applied in the formulation of novel equations for the determination of intermolecular distances, the distance covered before binding and the binding energy as shown in the appendix section.

3. MATERIALS AND METHODS

3.1 Materials

Human salivary alpha–amylase (EC 3.2.1.1) purchased from Sigma–Aldrich, USA. Hydrochloric acid, sodium hydroxide, and sodium chloride were purchased from BDH Chemical Ltd, Poole England, and insoluble potato starch was purchased from Sigma, USA. Tris was purchased from Kiran Light Laboratories, USA, 3, 5-dinitrosalicylic acid and maltose were purchased from Kem light laboratories, India. Sodium potassium tartrate tetrahydrate was purchased from Kermel, China, while calcium chloride was purchased from Lab Tech Chemicals, India. Distilled water was purchased from local market. All chemicals, their preparation and the enzyme were as reported elsewhere [25].

3.2 Methods

3.2.1 Assay of the enzyme

Stock solution of soluble potato starch was prepared by mixing 3 g in 50 ml of tris–HCl buffer at pH 7.4 subjected to heat treatment at 100°C for three minutes, cooled to room temperature, and final volume was made by topping the volume with buffer to 100 ml to give 30 g% as stock. Both substrate and enzyme were separately incubated at 37°C before assay. The final substrate concentration used for assay ranges from 5 to 9 g/L. A solution of the enzyme was prepared by dissolving 8.5 mg of the enzyme in 50 ml buffer containing first 5 mM calcium chloride and then 0.1% sodium chloride to give 0.17 g/l solution. Assay of the enzyme at 310.15 k using insoluble gelatinised potato starch as substrate is as previously described [25].

3.2.2 Determination of intermolecular distance at the beginning of intermolecular attraction

The equation for the calculation of intermolecular distance at the beginning of intermolecular attraction is given as:

$$R_0 = \acute{R}/\left(1 - \left(S_{\text{lope}}(1)/\left(S_{\text{lope}}(2)\right)^2\right)\right) \qquad (5)$$

Where Ŕ (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) and R_0 is the intermolecular distance at the commencement of onward motion towards the substrate due to initial attractive interaction. $S_{\text{lope}}(1)$ is the 1st slope from the plot of the square of effective collision frequency (ν) versus 1/ R_{int} ($R_{\text{int}} - \acute{R}$) and $S_{\text{lope}}(2)$ is the 2nd slope from plot of ν versus 1/R_{int} where, R_{int} is the concentration-dependent intermolecular distance. The frequency of collision, $\nu \approx 2\ \pi\ R\ D\ C_E$ 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 [26] at 310.15 K, weight average molecular mass of potato starch (7.73 exp (+7) g/mol) [27], and molar mass of virus given as 1.06 exp (+7)/ mol [26].

3.2.3 Determination of the terminal intermolecular distance, total translational and total electrostatic attractive energies

Equations (A.25/A.29), (4), and (A.35) were used for the calculation of terminal intermolecular distance, the total translational and total electrostatic attractive energies respectively.

3.3 Statistical Analysis

The mean and standard deviation (SD) were determined using Micro–soft Excel.

4. RESULTS AND DISCUSSION

4.1 Non–equilibrium Binding and Total Translational Energies

The main purpose of this section is to analyse and discuss the results (Table 1) against the backdrop of the use of alpha–amylase catalysed hydrolysis of gelatinised insoluble potato starch as a probable generalisable model for the determination and consideration of the importance of non–equilibrium binding energy. Therefore, literature pieces of information

regarding binding interaction need to be examined. Binding interactions between enzymes and substrates, directly at the site of chemical transformation and with other portions other than active site, carbohydrate binding domains for instance, have been shown to contribute to catalysis by being typically one of the rates–limiting steps preceding enzyme catalytic action during homogeneous reactions [28]. This is where carbohydrate binding modules become important. A carbohydrate binding module, restricted to carbohydrate–active enzyme, is defined as a contiguous amino acid sequence with a discrete fold having carbohydrate binding activity [29]. Their carbohydrate binding capacity is attributed, in part, to several aromatic amino acids that constitute the hydrophobic surface. As shown in Table 1, the total effective translational energy and in particular, the total electrostatic energy is higher in magnitude than the thermal energy. This is useful to the stability of the initial complex preceding the formation of activated complex through alternative root with lower energy barrier made possible by the enzyme in particular and water molecule (s). The purpose of the translational energy is to enable the molecules narrow the intermolecular distance so that with time they should be within reach of their intermolecular distance where electrostatic influence commences beginning with longer range interaction. This is the singular reason why higher velocity of amylolysis (Table 1) is observed at higher concentration of the substrate.

The mechanisms by which these noncovalent interactions can assist catalysis have been widely discussed, as follows. In the simplest scenario an enzyme can use binding interactions to localise the substrate to the active site. Binding interactions position the reactive portion of a substrate relative to active site functional groups and relative to other substrates [30–34]. This is preceded by the mutually advancing molecules in random motion though to a less extent in solution as against what is expected in the gas phase (Eq. (5)): At such intermolecular distance randomness is very minimal compared to bulk concentration dependent intermolecular distance, and it may mark the beginning of a mutual electrostatic perturbation beginning with greater effect of long–ranged attractive forces due mainly to the enzyme.

Concomitant with substrate binding is the displacement and exclusion of aqueous solvent from the active site and solvent exclusion may be important in modifying the electrostatic environment within the active site [35,36]. Indeed, solvent exclusion by substrate binding has been suggested to be important for catalysis in numerous enzymes [35–37]. The process of solvent exclusion may begin at the minimum intermolecular distance, $\mathfrak{R}_{ter}$ (expressed in Eqs. (A.25) / (A.29)), where, the highest electrostatic force is attained. The distance $\mathfrak{R}_{ter}$ though longer, is ≅ the sum of the hydrodynamic radii of the interacting molecules. Jencks et al. [30] realised that remote binding interactions can do more than provide for tight binding between substrate and enzyme. Reactions of bound substrates can be facilitated by the use of so-called ‘‘intrinsic binding energy’’ (restated in this research as non–equilibrium binding energy), which can pay for substrate desolvation, distortion, electrostatic destabilisation, and entropy loss [30,38–40].

The binding of cerium oxide nanoparticles, CNP to ds–deoxyribonucleic acid, ds–DNA occur by a nonbonding mode, an electrostatic interaction [23]. The implication of this statement is that electrostatic interaction is purely physical in nature which may not describe quantitatively an equilibrium state of a system. The domain of steric interaction occurs at Thymidine 12 (DT) moiety (the macromolecule to which the moiety belongs was not explicitly stated by the authors) [23]. The electrostatic interaction which was reported to have taken place between C2 of DT 12 (perhaps thymidine 12 of ds–DNA) and Ce atom and C6 of DT 12 and O atom of CNP at 2.466 and 2.312 Å may be the highest with the highest energy. But this is between two atomic moieties of electrostatically interacting macromolecule and inorganic compound unlike the much shorter interfacial distance (Table 1) reported for enzyme–substrate interaction in this research; whatever be the case, it needs to be noted that charge–charge (or polar) interaction has a range, beginning from an interparticle distance longer than the sum of their hydrodynamic radii. The short interfacial distance calculated in this research is due to the fact that the interaction is between a charged enzyme and weakly polar substrate under the prevailing pH. Although water molecule (s) may be displaced from one or both interacting particles, before binding can occur, it does not imply that the resulting complex remains dry; if not the complex may precipitate out of solution.

Table 1. Velocities of amylolysis and calculated physical parameters

Velocity*/mmol/L/mL.min	ξ_{eff} (*total)* /kJ/mol	$\xi_{(LS)}$ / kJ/mol	ΔR_{int} /exp (– 12) m
0.290 ± 0.03	2.726	3.154	7.570
0.300 ± 0.00	2.709	2.965	7.317
0.320 ± 0.00	2.702	2.887	6.860
$\approx 0.329 \pm 0.01$	2.691	2.754	6.672
$k_B\theta$	2.579 kJ/mol		

*The concentration of gelatinised water insoluble potato starch ranges between 6–9 g/L; * denotes data from the literature [25]; ξ_{eff} (total), $\xi_{(LS)}$ and ΔR_{int} are the total effective translational energy, total electrostatic energy, and the difference between two intermolecular distances, namely terminal intermolecular distance and the sum of the hydrodynamic radii of the binding molecules. The velocities of amylolysis and the physical parameters, energies and differences in two intermolecular distances were approximated to 3 decimal places so as to indicate any difference between magnitudes. The velocities of amylolysis were recorded as mean $\pm$ SD; k_B and θ are the Boltzmann constant and thermodynamic temperatures respectively. The maximum velocity of amylolysis at 310.15 k and pH = 7.4 is $\approx$ 0.457 mmol/mL.min and the concentration of human salivary alpha–amylase used is $\approx$ 2.78 exp (– 8) mol/L (8.5 mg solid–A103–1KU; Sigma–Aldrich, USA.)*

According to Schwan et al. [41] "the term intrinsic binding energy is not a molecular explanation for catalysis, but rather provides a conceptual framework for analysing the energetic of enzymatic catalysis. In this scenario the maximum binding energy is not realised in the ground state, because aspects of the bound state, such as restricted positioning of substrates, are energetically unfavorable relative to the interactions and freedom of motion in aqueous solution. However, changes associated with achievement of the transition state, such as charge and geometric rearrangements, and the formation of partial covalent bonds between positioned substrates, allow the binding energy to be more completely realised in the transition state". These views advanced by Schwan et al. [41] seem to imply that there is an increase in binding energy upon the formation of the activated complex. However, the report elsewhere is that a greater part of the intrinsic binding energy that results from noncovalent interaction of a specific substrate with the active site of the enzyme is expressed specifically at the transition state for the catalysed reaction *i.e*. an important part or rather a greater part of this binding energy may be utilised to provide the driving force for catalysis, so that the observed binding energy represents only what is left over after the utilisation [19].

The position held in this research is that intrinsic binding energy (once again renamed non–equilibrium energy) results from purely physical phenomenon, electrostatic, polar–polar, hydrophobic, van der Waal *etc* interactions intended to keep the substrate in place that will enable the enzymes' catalytic groups electronic configuration perturb the substrates' glycosidic bond (for the substrate, starch, as an example) leading to 1st, covalent bond breaking, before the formation of a new molecular electronic structure for bond formation in a manner seen as alternative root that lowers the activation energy for complex formation. Despite the release of water upon substrate binding, hydrolytic action requires the participation of a water molecule. If there is product binding, then product inhibition is implied but such binding is not chemical (covalent) in nature otherwise it should be irreversible as expected for a drug as inhibitor. Maltose for instance is not a substrate for alpha–amylase and as such it cannot be in a position to be hydrolysed any further.

4.2 Importance of Non–equilibrium Binding Energy

The importance of non–equilibrium binding energies is better corroborated or elucidated with examples of researches where binding affinity is regularly mentioned apart from the formation of encounter complex formation preceding enzyme–substrate complex formation used as model in this research. The webderimers (poly(amidoamine)) that are believed to possess binding affinity for nanoparticles for drug and gene encapsulation for drug transport and delivery [42] and cerium oxide nanoparticles (CNPs) for therapeutic applications for the cure of neurological oxidative stresses [23] requires information about the binding energy with both target and unintended target organ, tissue, cell *etc*. The 1st tier dendrimers with a common 1, 3,5–benzenetricarbonyl trichloride/trimesoyl chloride (TMC) core [42] can only exhibit its

scavenging activity against reactive oxygen species (free radicals which triggers carcinogenesis, cardiovascular disease *etc)* in an effective manner if they possess strong binding affinity (energy) that remains essentially irreversible.

The destruction of pathogens demands the production of drugs, following intense drug design, that target the pathogens, Covid–19 in particular. The interest lies essentially in the binding free energies that may have a link with the non–equilibrium binding energy. However, one must realise that attractive interaction between two different molecules may not necessarily translate into stronger binding if there is structural incompatibility, in line with the so–called "luck and key" complementarity. For an example research has shown that the anti–virus reversin showed the highest inhibitory efficacy against Covid–19, papain–like protease, as indicated by the ligand–protease binding energy determined by Mol soft pro analysis. The calculated inhibitory binding energy was −137.30 kJ/mol as compared with the tetrazapentadecanoate −129.57 kJ/mol, whereas remdesivir, pentagastrin, nitazoxanide and norfloxacin had a moderate antiprotease activity (> − 100 kJ/mol) [43]. The implication is that the non–equilibrium binding energy may be in the following order reversin > tetrazapentadecanoate > remdesivir, pentagastrin, nitazoxanide and norfloxacin. This is similar to nonviral enzyme–substrate interaction energy and catalytic activity given as follows. The Gibbs free energies were all negative, being higher in magnitude for methoxyresorufin (− 9.47 kcal/mol (theoretical) and − 8.80 kcal/mol (experimental)) than ethoxyresorufin (− 9.44 kcal/mol (theoretical) and − 8.51 kcal/mol (experimental)); all calculations were performed using the Insight II/Discover simulation package using the consistent valence force field [19]. The activity of the P450 1A1 was observed to be higher with ethoxyresorufin than with methoxyresorufin. What seems to play–out in the actions of the drug and substrate is not necessarily due to the non– equilibrium binding energy but subsequent events in the catalytic cycle, the chemical aspect, the breaking and making of new covalent bond; if the activation energy for covalent bond breaking is rather too high, a new bond may not be easily formed between the physically associated in–coming organic ligand (a drug or substrate) and the enzyme. The expectation for the drug is its inhibitory power via its irreversible new covalent bond formation with the pathogens' enzymes.

The comparison of the two docked resorufin substrates shows that the docking energy, which seems to mean the non–bond enzyme–substrate interaction energy, for 7–ethoxyresorufin (12.49 kcal/mol) is somewhat lower than that for 7–methoxyresorufin (15.0 kcal/mol). This may explain higher activity of P450 1A1 towards the former substrate [19]. This two literature information seem to suggest that a very strong affinity of a substrate to the enzyme expressed in terms of higher negative free energy of binding or non–covalent (non–bond) enzyme–substrate interaction energy does not necessarily translate into higher enzymatic activity. As shown in this research (Table 1) the highest total electrostatic, $\xi_{(LS)}$ (or the potential energy equivalent $-\xi_{(LS)}$) and translational energies were recorded for the lowest concentration of the substrate. In this research, the amylolytic activity of the amylase increased with increasing concentration of gelatinised insoluble potato starch, but the non–bond non–equilibrium binding energy was decreasing with increasing concentration of the substrate. It may seem to imply that "greater work analogous to higher activation energy" is involved where lower concentration of the substrate is available to the enzyme. This is further reflected in the higher translational energy at lower concentration of the substrates (Table 1). And, with regard to therapeutic application appropriate dose (concentration) of drug is therefore, essential aspect of health management.

Drug design preceding preclinical trials requires the much talked about molecular docking activities. Molecular docking is the computer–aided prediction of the bound geometry of two or more molecules. Molecules may be docked manually with the aid of computer graphics or automatically by using computer algorithms [44]. Molecular docking is a computational procedure that aims to predict the favoured orientation of a ligand to its macromolecular target (receptor), when these are bound to each other to form a stable complex [45].

This research seems to show how the non–equilibrium binding energy may be computed and it illustrates the fact that the energy cost is lower at higher concentration of the substrate (Table 1) which makes transition from mere encounter complex to any bullet–target complex formation faster such that higher concentration of product–destined enzyme–substrate could be formed. The goal of ligand–protein docking is to predict the predominant binding mode(s) of a ligand with

a protein of known three–dimensional structure [46,47]. Monika et al. [46] define molecular docking as the computational/ bioinformatic modeling of the structure of complexes formed by two or more interacting molecules.

The work of Rohs et al. [48] and Guedes et al. [49] reaffirms the notion that one of the implication of docking, the non–equilibrium binding energy, results from noncovalent interaction and one of the ultimate goal is to identify interaction with the lowest negative free energy of binding. Thus, what remains of utmost importance is a need for a non–equilibrium binding interaction (and cognate energy) between the bullet molecules, the drug, enzyme, *etc* and the target (supra) molecule, larger molecular weight substrate, pathogen like Covid–19, *Trypanosome brucei*, *Plasmodium falciparum etc*. This is with a view to inhibit the entry and/or replication of Covid–19 *in vivo* or *in vitro* and reproduction of any parasite in/on the body of human and nonhuman animals. Any failure of real–life docking procedure or process can be attributed to inadequate non–equilibrium binding interaction energy.

Where the non–equilibrium interaction energy ends functionally, bringing two different molecules into collisional contact, there the issue of structural complementarity begins which may be reflected in the magnitude of the negative free energy. In other words the non–equilibrium binding energy may be low but the structural complementarity favours binding with a resultant high magnitude of negative free energy or high association constant. Free energy as a thermodynamic parameter is not necessarily the same as non–equilibrium binding energy whose causative factors are not state functions. Replacing non–covalent intrinsic binding energy with non–equilibrium binding energy is informed by the view that internal energy and in particular, the two components of Gibbs free energy, the enthalpy and entropic energy, are intrinsic state quantities which can be used to describe quantitatively, an equilibrium state of a thermodynamic system; as state functions (SFs) they depend on the state at the beginning and the end of a process. On the other hand the work done (Eq. (A.35)) by the electrostatic field force in narrowing the gap between two particles that are within each others' electrostatic field as well as work done separately, against solvent resistance enhanced by thermal energy (Eq. (A.30)) cannot be described as intrinsic SFs since they are path functions.

5. CONCLUSION

The equations of non–equilibrium and translational energies were derivable. The binding interaction serves to initially fix the bullet molecule on or into the target (supra) molecule before the commencement of transition state complex formation. The non–equilibrium binding interactions of the bullet (drugs, substrate, *etc*) and target (receptors *e.g.* enzymes, pathogens such as Covid–19, *Plasmodium etc*) and the ultimate complex are likely to be stabilised against the thermal energy in furtherance of enzymatic and drug action since the electrostatic interaction energy is higher than thermal energy.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the author.

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COMPETING INTERESTS

Author has declared that no competing interests exist.

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APPENDIX

Determination of Terminal Intermolecular Distance, the Total Translational and Total Electrostatic Attractive Energies

The derivation of the equations for the terminal intermolecular distance of advancing molecules, the total translational and total electrostatic energies follows after a review of literature.

Review of Earlier Literature

Determination of the coefficient, ϕ of Coulomb equation and total work against solvent resistance

To begin with, one need to state that the interaction, the attractive case, between the enzyme and substrate, drug, ion, denaturant etc, are due to conservative field forces [50]. The interactions between the enzyme and the substrate, gelatinised insoluble potato starch, can be described mainly as ion-dipole and dipole-dipole interactions. The sum of these kinds of interaction energies is given as $\phi\, e^2/4\,\pi\,\varepsilon_0\,\varepsilon_r R_E$, the usual Coulombic equation where ϕ (this may be < or > 1) [24] is given as

$$\phi = 13.8564\,\mathfrak{r}_2^{½}(\pi\,\eta\,R_E\,P)^{½}\pi\,\varepsilon_0\,\varepsilon_r\left[R_{\text{int}}^3\left(R_{\text{int}}-\acute{R}\right)\right]^{½}/e^2 \tag{A.1}$$

Where, $\mathfrak{r}_2 = (M_S/M_2)^{½}/((M_S/M_2)^{½}+1)$ is a factor (a positive fraction) which takes into account the fact that the distance travelled by the enzyme is a fraction of the total distance ($\ell = R_{\text{int}} - (R_E + R_S)$) travelled between the particles; R_{int}, R_E, and R_S are the solution concentration dependent intermolecular distance, the hydrodynamic radius of the enzyme, and the hydrodynamic radius of the substrate; M_S and M_2 are the molar masses of the substrate and enzyme respectively; $\acute{R} = R_E + R_S$; η is the viscosity coefficient; P is the work down per unit time in overcoming solvent resistance; ε_0 and ε_r are the permittivity in vacuum and relative permittivity respectively.

$$P = 6\,\pi\,\eta\,R_E\,u_2\,R_{\text{int}}\,\nu \tag{A.2}$$

Where, u_2 is the translational velocity of the enzyme following attractive interaction with the substrate and ν is the frequency of collision.

$$\nu = 0.288675\left(\frac{P}{\mathfrak{r}_2\,\pi\,\eta\,R_E\,R_{\text{int}}\left(R_{\text{int}}-\acute{R}\right)}\right)^{½} \tag{A.3}$$

Thus,

$$P^{½} = \frac{6\,\pi\,\eta\,R_E\,u_2\,R_{int}\times 0.288675}{\left[\mathfrak{r}_2\pi\,\eta\,R_E\,R_{\text{int}}(R_{\text{int}}-\acute{R})\right]^{½}} \tag{A.4}$$

Substitution of Eq. (A.4) into Eq. (A.1) gives

$$\phi = 13.8564\,\mathfrak{r}_2^{½}(\pi\,\eta\,R_E)^{½}\frac{6\,\pi\,\eta\,R_E u_2 R_{\text{int}}\times 0.288675}{\left[\mathfrak{r}_2\pi\,\eta\,R_E R_{\text{int}}\left(R_{\text{int}}-\acute{R}\right)\right]^{½}}\pi\,\varepsilon_0\,\varepsilon_r\left[R_{\text{int}}^3\left(R_{\text{int}}-\acute{R}\right)\right]^{½}/e^2$$

Simplification gives

$$\phi \cong 24\,\pi\,\eta\,R_E\,u_2\,R_{\text{int}}^2\,\pi\,\varepsilon_0\,\varepsilon_r/e^2 \tag{A.5}$$

Next substitute Eq. (A.5) back into Eq. (A.1), simplify and rearrange to give

$$P \cong \frac{24^2\,\pi\,\eta\,R_E\,R_{\text{int}}u_2^2}{\mathfrak{r}_2 13.8564^2(R_{\text{int}}-\acute{R})}$$
$$\cong \frac{3\,\pi\,\eta\,R_E\,R_{\text{int}}\,u_2^2}{\mathfrak{r}_2(R_{\text{int}}-\acute{R})} \tag{A.6}$$

But, P is also given as shown in Eq. (A.1). Therefore, combining it and Eq. (A.6) gives first

$$P = 6\,\pi\,\eta\,R_E u_2\,R_{\text{int}}\,\nu = \frac{3\,\pi\,\eta\,R_E\,R_{\text{int}}\,u_2^2}{\boldsymbol{\tau}_2(R_{\text{int}}-\acute{R})} \tag{A.7}$$

Simplification of Eq. (A.7) gives

$$\nu = u_2/2\,\boldsymbol{\tau}_2\left(R_{\text{int}}-\acute{R}\right) \tag{A.8}$$

As in previous publication [24], ν is given as follows.

$$\nu = 2\pi\,\acute{R}\,D_{\text{E}}\,C_{\text{E}} \tag{A.9}$$

Thus, combining Eq. (A.8) and Eq. (A.9) gives

$$u_2 = 4\boldsymbol{\tau}_2\pi\,\acute{R}\left(R_{\text{int}}-\acute{R}\right)D_{\text{E}}C_{\text{E}} \tag{A.10}$$

However, u_2 is also given graphically as follows [24]:

$$u_2 = 2\boldsymbol{\tau}_2\left(S_{\text{lope}-1}\left(R_{\text{int}}-\acute{R}\right)/R_{\text{int}}\right)^{½} \tag{A.11}$$

Where, $S_{\text{lope}-1}$ is the 1st slope obtained from the plot of ν^2 versus 1/ R_{int} (R_{int} – $\acute{R}$), where R_{int} is = $\sqrt[3]{}$(exp (– 3)V_{s} (exp (– 3))/(n_{E} + n_{S}) N_{A}) and n_{E}, n_{S}, and N_{A} are the number of moles per cubic metre of the enzyme, substrate, and Avogadro's number respectively; exp (–3) is the conversion factor from litre to cubic metre.

Equation (A.11) expresses the translational velocity of the advancing enzyme towards to the larger substrate where the intermolecular distance is R_{int} for the concentration range of the substrate used for the assay. This does not exclude the concentration of the enzyme. Combining Eq. (A.10) and Eq. (A.11) gives 1st

$$4\boldsymbol{\tau}_2\pi\acute{R}\left(R_{\text{int}}-\acute{R}\right)D_{\text{E}}C_{\text{E}} = 2\boldsymbol{\tau}_2\left(S_{\text{lope}-1}\left(R_{\text{imt}}-\acute{R}\right)/R_{\text{int}}\right)^{½} \tag{A.12}$$

However, Eq. (A.12) where, D_{E} and C_{E} are the diffusion coefficient of the enzyme and the total number density of the enzyme respectively needs to be slightly modified by changing R_{int} to R_{eq} so as to quadratically determine the presumed intermolecular distance (R_{eq}, is the equivalent of R_0 determined graphically as stated in the method section) at the commencement of electrostatic attraction. Simplification gives

$$S_{\text{lope}-1} = 4\,\pi^2\,\acute{R}^2\left(R_{\text{eq}}-\acute{R}\right)R_{\text{eq}}\,D_{\text{E}}^2\,C_{\text{E}}^2 \tag{A.13}$$

Transformation into quadratic form gives

$$R_{\text{eq}}^2 - \acute{R}\,R_{\text{eq}} - \frac{S_{\text{lope}-1}}{4\pi^2\,\acute{\mathbf{R}}^2\,D_{\text{E}}^2\,C_{\text{E}}^2} = 0 \tag{A.14}$$

$$R_{\text{eq}} = \frac{\acute{\mathbf{R}} \pm \left(\acute{R}^2 + \frac{S_{\text{lope}-1}}{\pi^2\,\acute{R}^2 D_{\text{E}}^2\,C_{\text{E}}^2}\right)^{½}}{2} \tag{A.15}$$

The positive root of R_{eq} is given as

$$R_{\text{eq}} = \frac{\acute{\mathbf{R}} + \left(\acute{R}^2 + \frac{S_{\text{lope}-1}}{\pi^2\,\acute{\mathbf{R}}^2 D_{\text{E}}^2\,C_{\text{E}}^2}\right)^{½}}{2} \tag{A.16}$$

Equation (A.16) is an alternative to the equation for the determination of R_0 described in method section which is however, adopted for this research.

Substituting Eq. (A.10) into Eq. (A.7)/ ν and Eq. (A.7) to give respectively the work (ξ) done for overcoming solvent resistance and the corresponding power (*P*) in doing so.

$$\xi = 24\,\pi^2\,\eta\,R_E R_{\mathrm{eq}} \boldsymbol{\Upsilon}_2\,\acute{\mathrm{R}}\big(R_{\mathrm{int}} - \acute{\mathrm{R}}\big) D_{\mathrm{E}}\,C_{\mathrm{E}} \tag{A.17}$$

$$P = 48\,\pi^3\,\eta\,R_E\,R_{\mathrm{eq}} \boldsymbol{\Upsilon}_2 \big(R_{\mathrm{int}} - \acute{\mathrm{R}}\big)\big(\acute{\mathrm{R}}\,D_{\mathrm{E}}\,C_{\mathrm{E}}\big)^2 \tag{A.18}$$

At this juncture, there is a need to realise that Eq. (A.17) is based on Einstein-Stokes equation given as $6\,\pi\,\eta\,R_E\,u_2$ where u_2 is defined as Eq. (A.10) and should be seen as an initial burst velocity before terminal velocity is reached following initial acceleration and solvent resistance but tends to zero as the binding of the enzyme to the substrate occurs. This seems similar to the view that "as time increases, the rate coefficient decreases because the enzymes must diffuse to the substrate in order for reaction to occur" [51]. One should also bear in mind that there is always an initial velocity due to thermal energy. There is also a possibility of estimating the intermolecular distance at which the initial burst velocity occurs as follows: Based on the derived equation in the literature [24] in part the initial effective attractive energy ($\boldsymbol{\xi_{\mathrm{eff}}}$) is given as

$$\boldsymbol{\xi_{\mathrm{eff}}} = \frac{\left(\frac{\boldsymbol{\Upsilon}_2\,\ell}{m_2\,R_{\mathrm{eq}}} \pm \sqrt[2]{\left(\frac{\boldsymbol{\Upsilon}_2\,\ell}{m_2\,R_{\mathrm{eq}}}\right)^2 + \left(\frac{L}{R_{\mathrm{eq}}\,k_{\mathrm{B}}\,\theta}\right)^2 u_\theta^4}\right)\left(R_{\mathrm{eq}} k_{\mathrm{B}}\,\theta\right)^2}{(u_\theta\,L)^2} \tag{A.19}$$

Where m_2, k_{B}, L, u_θ, θ, and ℓ are the mass of a molecule of the enzyme, Boltzmann constant, (V exp (–6))^{⅓} where V is the molar volume of water converted to volume in cubic metre, initial velocity of the enzyme under thermal influence, thermodynamic temperature, and ($R_{\mathrm{eq}} - \acute{R}$) respectively.

Determination of terminal intermolecular distance at which maximum attractive interaction between the enzyme and substrate may occur

Before the derivation it is instructive to point out the fact that there are always several forces some of which are repulsive and other attractive; the attractive forces are of interest in this research. The charge–charge (or ion–ion), polar–polar (*e.g*. hydrogen bonding), polar–charge, non–polar–polar electrostatic interaction *etc* – the attractive type to be specific – are some of the examples that can be found in the literature [52]. In this research, enzyme–substrate complex is explored as a model for the determination of binding energy which always before any biochemical transformation formation. The long range forces bring the molecules closer to a shorter intermolecular distance where the short–ranged intermolecular forces become influential.

Combining Eq. (17) and Eq. (A.19) with modification (the replacement of R_{int} with $\mathfrak{R}_{(\mathrm{ter})}$), and after rearrangement one obtains,

$$\frac{24\,\pi^2\,\eta\;R_E\,\boldsymbol{\Upsilon}_2\,\acute{\mathrm{R}}\,(\mathfrak{R}_{(\mathrm{ter})} - \acute{\mathrm{R}})\,D_{\mathrm{E}}\,C_{\mathrm{E}}\,(u_\theta\,L)^2}{\mathfrak{R}_{(\mathrm{ter})}\,(k_{\mathrm{B}}\,\theta)^2} - \frac{\boldsymbol{\Upsilon}_2\,\ell}{m_2\,\mathfrak{R}_{(\mathrm{ter})}} = \sqrt[2]{\left(\frac{\boldsymbol{\Upsilon}_2\ell}{m_2\,\mathfrak{R}_{(\mathrm{ter})}}\right)^2 + \left(\frac{L}{\mathfrak{R}_{(\mathrm{ter})} k_{\mathrm{B}}\,\theta}\right)^2 u_\theta^4} \tag{A.20}$$

Squaring and rearrangement gives

$$\big(\mathfrak{R}_{(\mathrm{ter})} - \acute{\mathrm{R}}\big)^2 \left(\left(\frac{24\,\pi^2\,\eta\;R_E\,\boldsymbol{\Upsilon}_2\,\acute{\mathrm{R}}\;D_{\mathrm{E}}\,C_{\mathrm{E}}\,(u_\theta\,L)^2}{(k_{\mathrm{B}}\,\theta)^2}\right)^2 - \frac{48\,\pi^2\,\eta\,R_E\,\boldsymbol{\Upsilon}_2^2\,\acute{\mathrm{R}}\,D_{\mathrm{E}}\,C_{\mathrm{E}}(u_\theta\,L)^2}{(k_{\mathrm{B}}\,\theta)^2\,m_2}\right) = \left(\frac{L\,u_\theta^2}{k_{\mathrm{B}}\,\theta}\right)^2 \tag{A.21}$$

Simplification gives

$$\big(\mathfrak{R}_{(\mathrm{ter})} - \acute{\mathrm{R}}\big)^2 \left(\big(24\,\pi^2\eta\,R_E\boldsymbol{\Upsilon}_2\,\acute{\mathrm{R}}\,D_{\mathrm{E}}\,C_{\mathrm{E}}(u_\theta\,L)^2\big)^2 - \frac{48\,\pi^2\eta\,R_E\,\boldsymbol{\Upsilon}_2^2\,\acute{\mathrm{R}}\,D_{\mathrm{E}}\,C_{\mathrm{E}}(u_\theta\,L)^2(k_{\mathrm{B}}\,\theta)^2}{m_2}\right) = \; (L\,u_\theta^2 k_{\mathrm{B}}\theta)^2 \tag{A.22}$$

Before proceeding further, it is necessary to state that combining Eq. (A.17) and Eq. (A.19) suggests that there is a terminal velocity which occurred at a given terminal intermolecular distance. This distance covered shortly before collision with the substrate $\mathfrak{R}_{(\mathrm{ter})} - \acute{\mathrm{R}}$, is given as:

$$\Re_{(\mathrm{ter})} - \acute{\mathrm{R}} = \sqrt[2]{\frac{\left(L\,u_\theta^2\,k_\mathrm{B}\,\theta\right)^2}{\left(\left(24\pi^2\,\eta\,R_E\boldsymbol{\Upsilon_2}\,\acute{\mathbf{R}}\,D_\mathrm{E}\,C_\mathrm{E}(u_\theta\,L)^{\mathbf{2}}\right)^2 - \frac{48\,\pi^2\,\eta\,R_E\,\Upsilon_2^2\,\acute{\mathbf{R}}\,D_\mathrm{E}\,C_\mathrm{E}\,(u_\theta\,L)^2(k_\mathrm{B}\,\theta)^2}{m_2}\right)}} \tag{A.23}$$

The terminal velocity shortly before collision with the substrate is expectedly lower than at intermolecular distance longer than terminal intermolecular distance, $\Re_{\,(\mathrm{ter})} - \acute{\mathrm{R}}$. Equation (A.23) can be simplified (this is with the understanding that $6\,\pi\,\eta\,R_\mathrm{E} = k_\mathrm{B}\theta\,/D_\mathrm{E}$) to give.

$$\Re_{(\mathrm{ter})} - \acute{\mathrm{R}} = \sqrt[2]{\frac{(L\,u_\theta)^2}{\left(\left(4\,\pi\,\boldsymbol{\Upsilon_2}\,\acute{\mathbf{R}}\,C_\mathrm{E}\,\boldsymbol{L^2}\,u_\theta\right)^2 - \frac{8\,\pi\,k_\mathrm{B}\,\theta\,\Upsilon_2^2\,\acute{\mathbf{R}}\,C_\mathrm{E}\,L^2}{m_2}\right)}} \tag{A.24}$$

Ultimately,

$$\Re_{(\mathrm{ter})} = \sqrt[2]{\frac{(L\,u_\theta)^2}{\left(\left(4\,\pi\,\boldsymbol{\Upsilon_2}\,\acute{\mathbf{R}}\,C_\mathrm{E}\,\boldsymbol{L^2}u_\theta\right)^2 - \frac{8\,\pi\,k_\mathrm{B}\,\theta\,\Upsilon_2^2\,\acute{\mathbf{R}}\,C_\mathrm{E}\,L^2}{m_2}\right)}} + \acute{\mathrm{R}} \tag{A.25}$$

Meanwhile, there are stages during the advance of the enzyme towards the substrate viz: initial attraction leading to increase in translational velocity from the initial bulk velocity under the effect of thermal energy; this takes awhile after covering a distance while in transit. But soon there is a decline in velocity to a value < than the peak value but > value due to thermal energy. There are two peak values for any substance. The first is the peak value of a soluble substance in solid state or concentrated state just introduced into a compatible solvent. Separation of the solute molecules increases rapidly, reaching a peak velocity (u_θ) but soon decreases due to solvent resistance [21] to a terminal velocity (u_θ^{ter}) when all the solute molecules have achieved a total uniform distribution (or uniform concentration in the solution). Thus, in a purely thermal environment in solution the "thermal field force", TFF [21], $k_\mathrm{B}\theta/L = 6\pi\eta R_E u_\theta^{\mathrm{ter}}$. If the distance covered before reaching the terminal velocity is *x* for instance, the work is $k_\mathrm{B}\theta x/L = 6\pi\eta R_E u_\theta^{\mathrm{ter}} x$.

However, when solutes reach the region of mutual electrostatic perturbation (attractive case in this research), there is the first stage of events, the initial increase in translational velocity to a peak value (now designated as $u_{2\mathrm{e}}$) which shortly thereafter decreases to a terminal velocity, $u_{2\mathrm{e}}^{\mathrm{ter}}$ (the 2nd event) which in turn tend to a lower velocity (3rd event) until zero value (the 4th event) when the enzyme binds to the substrate.

Another approach in the determination of terminal intermolecular distance $\Re_{\,(\mathrm{ter})}$ (the unknown to be determined), shortly before collision of the enzyme with substrate is based on the proposition (or rather postulation) that the work against solvent resistance in the course of thermal energy driven motion outside electrostatic influence is equal to work done against solvent resistance in the course of electrostatically driven motion. Thus,

$$\frac{6\,\pi\,\eta\,R_\mathrm{E}\,D_\mathrm{E}\left(R_\mathrm{eq} - \Re_{(\mathrm{ter})}\right)}{L} = 24\,\pi\,\eta\,R_E\boldsymbol{\Upsilon}_2\pi\,\acute{R}\left(R_\mathrm{eq} - \acute{R}\right)D_\mathrm{E}\,C_\mathrm{E}\left(\Re_{(\mathrm{ter})} - \acute{R}\right) \tag{A.26}$$

Simplification and rearrangement gives

$$\frac{R_\mathrm{eq} - \Re_{(\mathrm{ter})}}{L} = 4\boldsymbol{\Upsilon}_2\pi\,\acute{R}\left(R_\mathrm{eq} - \acute{R}\right)C_\mathrm{E}\left(\Re_{(\mathrm{ter})} - \acute{R}\right) \tag{A.27}$$

$$\Re_{(\mathrm{ter})}\left(\frac{1}{L} + 4\,\boldsymbol{\Upsilon}_2\,\pi\,\acute{R}\left(R_\mathrm{eq} - \acute{R}\right)C_\mathrm{E}\right) = \frac{R_\mathrm{eq}}{L} + 4\,\boldsymbol{\Upsilon}_2\,\pi\,\acute{R}^2\left(R_\mathrm{eq} - \acute{R}\right)C_\mathrm{E} \tag{A.28}$$

$$\Re_{(\mathrm{ter})} = \frac{(R_\mathrm{eq}/L) + 4\,\boldsymbol{\Upsilon}_2\pi\,\acute{\boldsymbol{R}}^2\left(R_\mathrm{eq} - \acute{\boldsymbol{R}}\right)C_\mathrm{E}}{\left(\frac{1}{L} + 4\,\boldsymbol{\Upsilon}_2\pi\,\acute{\boldsymbol{R}}\left(R_\mathrm{eq} - \acute{\boldsymbol{R}}\right)C_\mathrm{E}\right)} \tag{A.29}$$

Equations (A.25) and (A.29) give the same result with different values of R_{int}. In other words the same values of $\Re_{(\mathrm{ter})}$ can be obtained if R_{eq} is replaced with R_{int} in Eq. (A.29).

Having established the value of intermolecular distance, $\Re_{(ter)}$ being the value at which the terminal velocity was reached, the work, ξ_θ down against solvent resistance outside the electrostatic field can be given as

$$\xi_\theta = 2\,\Upsilon_2\,k_B\theta\,(R_{int} - \Re_{(ter)})\,/L \tag{A.30}$$

Where, R_{int} and $\Re_{(ter)}$ have been defined in the text and Eq. (A. 25/29) respectively. There is also, work (ξ_{elect}) down against solvent resistance within the mutual electrostatic field of the enzyme and substrate, given as:

$$\xi_{elect} = 2\,\mathcal{F}_{elect}\,\Upsilon_2\left(\Re_{ter} - \acute{R}\right) \tag{A.31}$$

Where, ξ_{elect} is the total electrostatic force of attraction. The total work, ξ_{total} against solvent resistance is given as:

$$\xi_{total} = 48\,\pi^2\,\eta\,R_E\,\acute{R}\,D_E\,C_E\,\Upsilon_2^2\,(R_{int} - \acute{R})^2 \tag{A.32}$$

The product of 2 Υ_2 appears in Eqs (A.30), A. (31), and (A.32) and as such it can be eliminated from the equation below. Thus,

$$2\,k_B\,\theta\,\Upsilon_2\,(R_{int} - \Re_{(ter)})\,/L + 2\,\mathcal{F}_{elect}\,\Upsilon_2\left(\Re_{ter} - \acute{R}\right) = 48\,\pi^2\,\eta\,R_E\,\acute{R}\,D_E\,C_E\,\Upsilon_2^2\,(R_{int} - \acute{R})^2 \tag{A.33}$$

Equation (A.33) is a result of reintroducing R_{int} in place of R_{eq}.

$$\mathcal{F}_{elect} = \frac{24\,\pi^2\,\eta\,R_E\,\acute{R}\,D_{E2}\,C_E\,(R_{int} - \acute{R})^2 - \frac{k_B\,\theta}{L}\,(R_{int} - \Re_{ter})}{\Re_{(ter)} - \acute{R}} \tag{A.34}$$

The total electrostatic energy ($\xi_{(LS)}$) of attraction the long–ranged electrostatic attractive energy which occurs earlier at a longer intermolecular distance plus short–ranged energy which occurs latter at a shorter intermolecular distance is given as:

$$\xi_{(LS)} = \mathcal{F}_{elect}\,R_0 \tag{A.35}$$

One can conclude this derivation with the opinion that as the intermolecular distance closes up the force of attraction being the sum of weak and strong forces in particular becomes stronger but not without solvent resistance. What is often called long–ranged and short–ranged electrostatic interaction, requires a look at the following ratios $a_0\,/r_m > R_0\,/R_E \gg R_0\,/R_S$ ($\approx 48.091 > \approx 39.680 \gg \approx 2.943$): Where, a_0 and r_m are the Bohr radius for hydrogen (0.529 Å) atom and mass radius of hydrogen atom, assumed to be 1.1 fm. By putting the size of hydrogen atom into consideration one can definitively conclude that it has the longest long–ranged electrostatic force of attraction followed by the enzyme and the substrate with a very short range. Thus considering the size of the enzyme relative to the hydrogen atom, the intermolecular distance equal to R_0 may not be too long; but this remains a tentative assumption.

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