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The Denaturation and Renaturation Kinetics of β-Galactosidase by Chemical Denaturants in The Presence of Galactose, a Product Inhibitor

Okechukwu Nwamba[1]

1 University of Idaho

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Abstract

In the present report, a quantitative characterization predicting functional allosteric states as a function of chemical denaturants (urea and Guanidine Hydrochloride) and ligand (galactose) concentrations has been presented for β-Galactosidase. The exact calculations predicting the apparent inactivation rate constant, *A*, and the product formed at infinite observable time, $[P]_{\infty}$, both as functions of protein unfolding studies in the presence of ligands are derived with implications of these derivations. These derivations, which are thermodynamic parameters, ultimately represent the apparent rate of enzyme/protein inactivation when an unfolded/unfolding protein interacts with ligand modifiers (*A* is the rate at which the protein is inactivated under such conditions) and the accompanying $[P]_{\infty}$ which can translate to K_{cat} (catalytic constant), K_c the enzyme turnover number which can translate – for example – to enzyme shuttling activities across membrane barriers, or even conversion efficiency from one enzyme form to the other on ligand binding to the unfolding/unfolded protein form as a result of allostery. Prior to these derivations, it has been impossible to quantify and characterize allosteric transition conditions via mathematical calculations. Simulation-based multiscale methods have been the way to make rough estimates-in-the-ball-park predictions of allosteric characterizations for quantitative purposes. Even though these derivations were made for β-galactosidase using chemical denaturants as the unfolding agents and galactose as the ligand modifier, it is expected that these equations will cut through different proteins, unfolding conditions, and ligand modifiers.

Okechukwu Charles Nwamba, Ph.D.

Department of Chemistry, University of Idaho, 875 Perimeter Drive, MS 2343, Moscow, ID 83844-2343, USA

Email: nwam5645@vandals.uidaho.edu; charlesnwamba1@gmail.com

Introduction

In textbooks and journal publications on enzyme-substrate interactions, enzyme-ligand interactions,[1][2][3][4] and at least,

in the last three decades, enzyme-denaturant interactions,[5][6][7][8][9][10][11][12] have been extensively investigated as models for protein stability studies. On the other hand, in modern biochemistry and biophysics, protein stability (determined by elucidating the unfolding/refolding kinetic and thermodynamic profiles of the protein during its denaturation. Protein denaturation can be induced using chemical denaturants such as urea and Guanidine Hydrochloride or physical denaturants such as thermal and UV denaturation protocols) and ligand interaction studies – on the same molecule – are of high interest.[13][14][15][16][17] Some interest areas include molecular recognition such as elucidation of molecular recognition patterns, mechanisms of action even amongst functionally similar drugs, and ligand binding modes as a function of denaturant – and varying ligand concentrations (and vice versa). This type of protein stability – ligand interactions are particularly important in membrane environments where membrane binding/interaction to a protein could induce partial unfolding of the protein while, at the same time, the protein is equally interacting with ligands. The consequences of this type of three-body interaction are important in protein misfolding diseases such as prion diseases, Alzheimer's, Parkinson's, transmissible spongiform encephalopathies, familial amyloid polyneuropathy, Huntington's disease, type II diabetes, and sickle cell diseases.[18][19][20][21][22][23][24][25] [26][27][28][29][30][31] Also, these kinds of three-body interactions are very important as regulatory and multitasking agents in health and disease states, when the proteins of interest are, for example, disordered, moonlighting, or morpheein proteins.[32][33][34][35][36][37] These type of proteins will always involve the use of disordered protein regions,[35][36] different faces of the same proteins (moonlighting proteins),[32][33][34] or different building units of the same proteins (morpheein proteins),[37][38] – all always involving ligands and transitionary unfolded states.

Other interests in protein-denaturant-ligand interaction include screens for drug-target identification, structural genomics or proteomic investigations, formulation of biopharmaceutical proteins, protein engineering, and drug screening.[39][40][41] With respect to drug screening – for example – such is done to discover new protein-ligand interactions, protein-cofactors interactions, buffers, and additives that maximize protein stability, during thermal denaturation by hinging on the increased protein stability that is typical for ligand binding.[13][42][43][44] Other important applications include elucidation of signal transduction pathways, or important players in signal transduction.[45][46][47][17] Thus, these three-body interactions are very important in pharmacology, pharmacokinetics, toxicology, and drug development, to mention a few.

Even though thermodynamic parameters (such as changes in Gibb's free energy, ΔG; reaction enthalpy changes, ΔH; differences in chemical potentials, $\Delta\mu$; etc.) for protein stability during denaturation, and in the presence of protein-ligand interactions, have been reported in the literature[17][18][48][49][50][51], there have been no calculations, such as those carried out by Tsou and colleagues for protein-denaturant interactions nearly twenty-five years ago,[12] to determine kinetic parameters such as the Apparent inactivation rate constants (A) and the product infinity $[P]_{\infty}$ for protein denaturation (protein stability) studies during protein-ligand interactions. These parameters and their derivatives are important for estimating protein turnover numbers in the presence of competing inhibitory/regulatory proteins and activator molecules (for example, when determining the nuclear-to-cytoplasm turnover of protein shuttling activities in the presence of an enzyme-regulator protein –which could be viewed as inhibitory – and varying activator molecules,[52][53][54]), protein shuttling activities from outside the cell to the interior of the cell (e.g., as the rate at which substrate – for example, glucose

– is phosphorylated and shuttled inwards simultaneously, across the membranes, to a phosphorylated glucose compound inside the cell[55][32][56]) and for predicting requisite ligand concentrations during protein denaturation (protein stability) studies.

Another important aspect is the enzyme catalytic activity when unfolded (such as protein interaction with membranes, which can cause unfolding), but in the presence of a modulating factor, e.g. a ligand (such as cofactors, specific ligands required for the protein activity, other ligands within the vicinity of the protein). These three-body interactions can substantially alter not only the kinetic and catalytic properties, but also the mechanism of action, of the said protein of interest. The difficulty in the derivation of these two kinetic parameters, and other intervening kinetic parameters during these reactions, lies with the conformational changes, occurring most frequently before significant protein unfolding (then refolding when ligand interaction is factored in), and subsequently occurring in parallel with significant protein unfolding.[57][58][59] These kinetic parameters fluctuate with each denaturant and or ligand concentration,[12][57] and could be seen as akin to hitting a moving target.

Some years back, some works on protein-ligand interactions as a function of denaturant concentrations (protein stability studies) were published.[60][61] Important in those works were the changing modes of inhibitor interactions with the enzyme as a function of inhibitor concentrations at steady denaturant concentrations (in other words, varying inhibitor concentration during protein stability studies). In this type of study, the product concentrations at infinity, $[P]_{\infty}$, are no longer subject to the conventional Michaelis-Menten (MM) equation, yet they follow the pattern of the MM equation. In this report, a derivation of the various thermodynamic and kinetic parameters *en route* the derivation of the apparent inactivation rate constant, [A], and product formed at infinite time, $[P]_{\infty}$. in other words, for every protein stability and protein-ligand study, the end game is always the output of the function desired for that protein such as catalysis (thus, the product formed at infinite time, $[P]_{\infty}$) when the enzyme/protein is experiencing apparent inactivation (apparent because of the partial stabilization of the protein by the ligands during the denaturation).

Materials and Methods

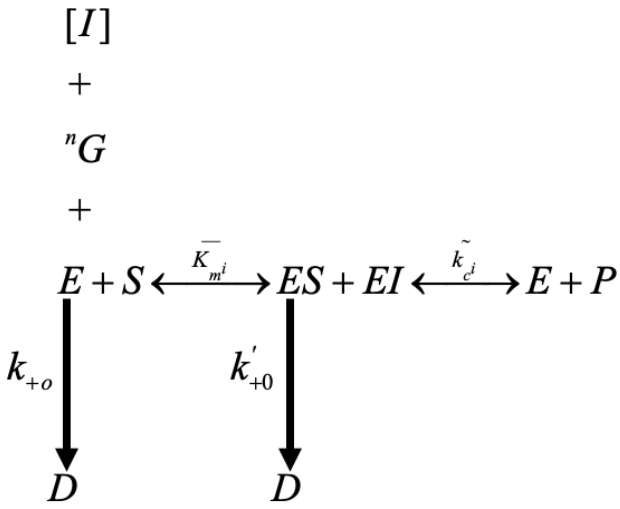
The materials and methods employed for the isolation, purification, characterization, and kinetic analysis of b-galactosidase form the black bean, *Ketsingiella geocarpa* in the presence of ligands (lactose, galactose and glucose) only,[62] ligand (galactose) and urea (denaturant),[60] and ligand (galactose) and GdnHCl (denaturant),[61] have been previously reported. Briefly, the germinated beans were washed, and the beans testa were removed to leave behind the cotyledons, which were subsequently frozen. The frozen cotyledons were then homogenized in ice-cold 10 mM Tris/HCl buffer pH 8.0, containing 0.8% Triton X-100.[63] Afterwards, the homogenate containing the crude enzyme-chaff mixture was strained through a double-layer cheese cloth to separate the chaff from the crude enzyme. The supernatant as the crude enzyme was loaded onto a DEAE-cellulose column (2.5/25 cm) equilibrated with 10 mM Tris-HCl buffer, pH 8.0, to remove the ammonium sulphate. The fractionation of the purified enzyme into its active constituents, enzyme concentration using a Sephadex G-100 column, protein estimation using Folin Ciocalteau (Lowry method) and enzyme assays using p-Nitrophenyl β-D-galactopyranoside (PNPG) as a substrate have been rigorously explained

elsewhere.[62] On the other hand, the Effect of substrate (PNPG) concentration on urea, and GdnHCl inactivation of β-galactosidase in the presence and absence of galactose (inhibitor/modifier) have also been reported in the literature.[60][61]

Theory of Kinetic Analysis

It was deduced that the mode of enzyme inhibition and reactivation in the presence of denaturant and varying ligand concentrations, even for given ligand concentrations, varied as a function of the chemistry of the denaturant employed – GdnHCl vs Urea. Thus, the kinetic analysis of the interaction between these three bodies (protein, ligand, and denaturant) has been carried out by extending the kinetic analysis of the ligand and enzyme interaction studies by Xiao *et* al.[64] and Wang *et al.*[12] In the present analysis that theoretically reproduces the experimentally calculated values of $[P]_\infty$ and *A* reported earlier.[60][61]

The kinetic analysis is derived starting with the terms shown in **Scheme 1**. In this case, the terms *E* and *S* represent the native enzyme and substrate respectively, while *ES* is the Enzyme-Substrate complex. The term *D* denotes denatured enzyme state, *P* represents products formed, k_0 is the denaturation rate constant for the free enzyme, $k_0^{'}$ is the denaturation rate constant for the enzyme-substrate complex, k_{+0} is the first-order inactivation rate constant for the free enzyme concentration ($[E]_o$) and can be thought of as the microscopic or intrinsic rate constant of the free enzyme, irrespective of the numerous enzyme complexes along the denaturation/renaturation pathways (see the **Appendix section** for more of these enzyme-bound complexes). Likewise, the corollary term $k_{+0}^{'}$ is the first-order inactivation rate constant for the enzyme-substrate (*[ES]*) complex and can be thought of as the microscopic or intrinsic rate constant of the enzyme-substrate (*[ES]*) complex, independent of the various bound complexes to the *[ES]* form. The terms, $\tilde{K}_{m^i}$ and $\tilde{k}_{c^i}$ are the Michaelis Menten constant and the turn-over number respectively of the enzyme-catalyzed reaction in the presence of denaturant and inhibitor. For a complete description of the entire parameters, kinetic and thermodynamic, given or derived, the reader is referred to the **Appendix section**.



Scheme 1

As in classical enzymology, the kinetic studies of the enzyme inactivation in the presence of ligands were done at saturating substrate concentration.[12][65] Thus, employing the conditions for the Michaelis – Menten derivation, the substrate concentration, [S], is regarded as being constant during the reaction and thus set as equal to its initial value in the derivation of the integrated rate equations such that:

$$[S] = [S]_0$$

$\therefore$ the substrate conc., $[S]$ during the reaction is regarded as same with its inital conc. $[S]_0$

And

$$\mathrm{E}_{Total}, E_T = ES + EI$$

If we asssume a first order reaction, such that

$$[S] \gg [E_0]$$

and or

$$[S] \gg [E_T]$$

since at

$$t = 0; [E_T] = [E_0]$$

Then

$$[E_T] = [E_0]e^{-At} \quad \text{.....(1)}$$

Thus,

$$([ES] + [EI]) = [E_0]e^{-At} \quad \text{.....(2)}$$

Thus, the rate of enzyme inactivation is expressed as:

$$\frac{dy}{dx} = \frac{d([ES]+[EI])}{dt} = \frac{d\left([E_0]e^{-At}\right)}{dt} = A[E_0] = A[E_T] \quad \text{.....(3)}$$

$$= A[E_T]$$

$$= A([ES]+[EI]) \quad \text{.....(4)}$$

Thus, from

$$S + E_T \xrightarrow{A} P \quad \text{.....(5)}$$

$$A[P] = \frac{d[P]}{dt} = v_0 = -\frac{d[S]}{dt} = -\frac{d[E_T]}{dt} \quad \text{.....(6)}$$

Where

$$A = \frac{k_{+0}.\overline{Km}.Km'.Ki + \left([S]Km'.Ki + [I]Km\right).k'_{+0}}{\overline{Km}.Km'.Ki + [S]Km'.Ki + [I]Km} \quad \text{.....(7)}$$

A is the apparent inactivation rate constant of the enzyme denaturation during catalysis in the presence of the inhibitor. All the kinetic derivations are functions of a given denaturant concentration ([Urea] or [GdnHCl]) and varying ligands (in this case, galactose concentrations).

Employing the boundary conditions in Equation 1 where $t = 0$; $[E_t] = [E_0]$, we have

$$A[P] = v_0$$

$$[P] = \frac{v_0}{A}(1 - e^{-At})$$

Then, at boundary conditions of infinite time when observation time doesn't change product yield; *i.e.*, *at* $t = \infty$

$$[P] = [P]_\infty$$

So that

$$[P] = \frac{v_0}{A}(1 - e^{-At}) = [P]_\infty(1 - e^{-At})$$

$$[P]_\infty = \frac{v_0}{A} \qquad \text{.....(8)}$$

$$= \frac{2.[E].\overline{kc}[S]_0[I]}{k'_{+0}.\left(\overline{Km}.[\mathrm{I}] + Km'.Ki[S]_0\right) + k_{+0}.\overline{Km}.Km'Ki} \qquad \text{.....(9)}$$

An exhaustive derivation of the steps, the assumptions made and various calculations required to obtain the apparent inactivation rate constants, and product formed at infinite time, during protein unfolding studies in the presence of ligands can be found in the Electronic Supplementary Information (ESI) section.

Results and Discussion

A plot of the product formed at time *t*, $[P]_t$ against the given time, *t* up to infinite time, $[P]_\infty$ (when the product concentration formed does not change again with observable time) is presented in **Figure 1**. As earlier explained in the previous reports,[60][61] and just as a refresher, the introduction of the chemical denaturants, urea or GdnHCl decreased the $[P]_\infty$. A linear regression plot of $\ln([P]_\infty - [P]_t)$ vs. time *t* will give a negative slope known as A (**Figure 2**), the apparent inactivation rate of the enzyme. It was shown that the substrate concentration, [S] had no protective effect on the free enzyme when bound to the enzyme to form an enzyme-substrate, [ES] complex, since the plot of *A* vs [S] was of zero order. As such, $A = k_{+0} = k'_{+0}$, where k_{+0} and k'_{+0} are the microscopic rate constants for the free enzyme and the enzyme-substrate complex respectively as previously explained.

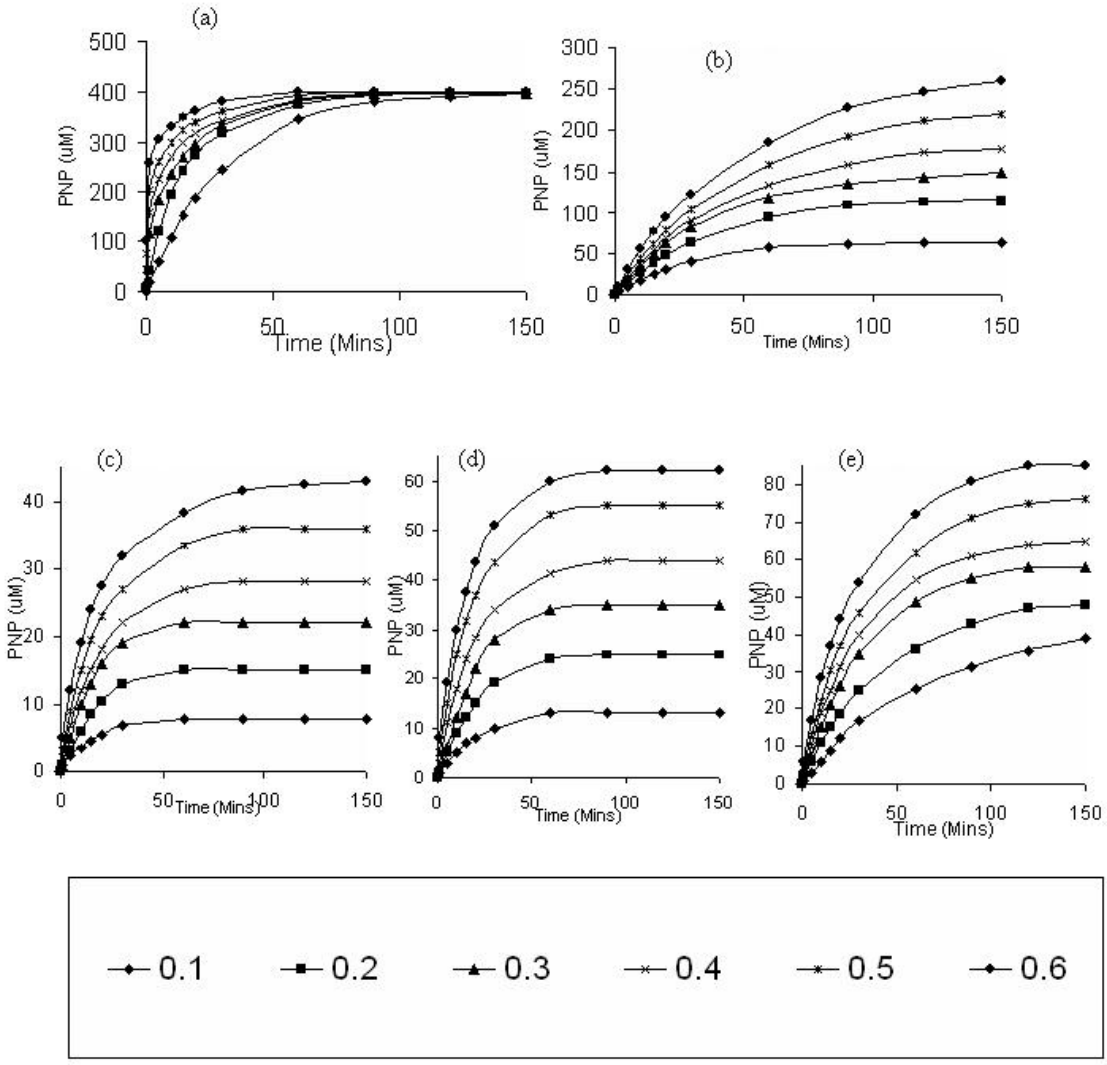


Figure 1. Kinetics of the inactivation of β-galactosidase in the absence and presence of 3M GdnHClat 50 ^{O}C, pH 4.5, and at different concentrations of substrate, PNPG, [0.10 - 0.60] a: In the absence of GdnHCl (No GdnHCl); b: in the presence of 3M GdnHCl; c: in the presence of 3M GdnHCl and 5mM galactose; d: in the presence of 3M GdnHCl and 10mM galactose; e: in the presence of 3M GdnHCl and 20mM galactose. Adapted from [61].

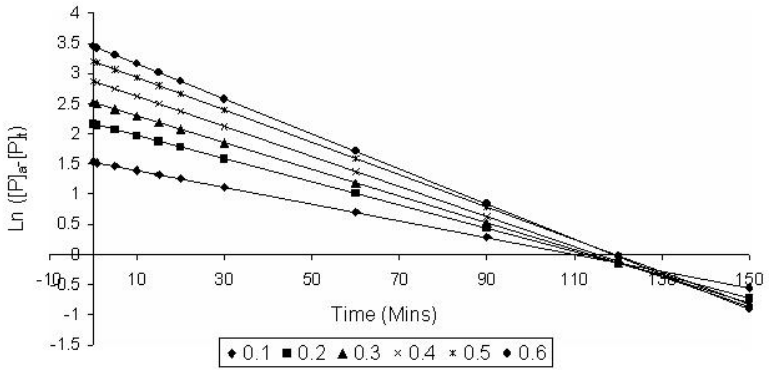


Figure 2. Semi logarithmic plot of P(μM) vs time (t) of data in Fig. 1c: ln([P] $_{a}$-[P]$_{t}$) vs t for 3M GdnHCl, 5mM Galactose. N.B: [P]$_{a}$ = [pNP]; [P]$_{t}$ = [pNP]$_{t}$. Adapted

from [61].

However, on introduction of the galactose concentrations to the protein denaturation study, then $A \neq k_{+0} \neq k'_{+0}$ so that the galactose protects the free enzyme but not the enzyme-substrate, [ES] complex (**Figure 3**). This is because once the [ES] complex is formed, the reaction goes downhill to form the product and the free enzyme, which can then be bound, stabilized, and renatured by the galactose prior to substrate binding and the entire cycle repeats itself. When various parameters such as K_i values (values obtained from [62]), K_m values in the presence of the denaturants, and inhibitors, respectively, kc (turnover number in the presence of denaturant only), k_{+0}, and k'_{+0} (values obtained from [60][61]) were inputted into equations 7 and 9, the values of A ranging from 0.0135 – 0.0380 s^{-1} were obtained for β-galactosidase, as previously reported. The same is true for the $[P]_\infty$ obtained from equation 9.[60][61]

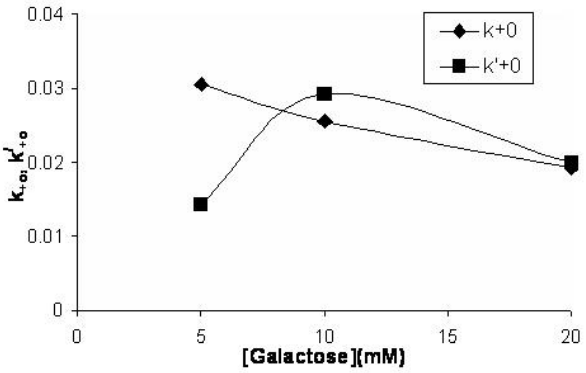


Figure 3. Plot of k_{+o}, k'_{+o} vs [Galactose]. k_{+o} and k'_{+o} are the microscopic inactivation rate constants for the free enzyme and enzyme-substrate complex respectively. Adapted from [61].

Interestingly, on introduction of the inhibitor (galactose in this case), and at increasingly higher concentrations, to the protein denaturation studies, the concept of enzyme memory or hysteresis is introduced. These phase-shifting and the consequences of these phase-shifting mechanisms as regard the derivation of equations that predict accurately the *A* and $[P]_\infty$ in the presence of chemical denaturant and galactose (inhibitor) concentration.

In this report, it is shown that irrespective of the type of chemical denaturant employed and varying ligand inhibition binding mode required to reactivate the protein via, for example, the morpheein allosteric model,[60][61][66] it has been computed that the same equations serve to determine, via calculations, the product infinity ($[P]_\infty$) and apparent inactivation rate constants, *A*, in the presence of denaturants and inhibitors. These modes of interaction (dissociating/dissociated protein-ligand interaction) have been suggested to be responsible for small-molecule perturbation of quaternary structure equilibria,[67][68] leading to various responses such as drug-induced side

effect,[66] **Ref** disease manifestation leading to the consequent designing of bioactive molecules, such as in therapeutics.[68]

In this regard, we derive the kinetic and thermodynamic parameters that ultimately represent the apparent rate of enzyme/protein inactivation when an unfolded/unfolding protein interacts with ligand modifiers and the accompanying $[P]_{\infty}$ which can translate to K_{cat} (catalytic constant), enzyme turnover number which can translate to enzyme shuttling activities across membrane barriers, for example, and or even conversion efficiency from one enzyme form to the other on ligand binding to the unfolding/unfolded protein form.

Thus, via ligand (galactose in this case) binding to partially unfolded enzyme states, we are calculating ligand concentrations required to effect allosteric shifts from one enzyme form to another with different catalytic properties.[69][38][68] The Morpheein model, for example, is a type of allostery.[69][38] By virtue of these exact calculations, we have provided an answer to a common query in the community as represented by the concern posed in the literature: "*despite significant advances, the quantitative characterization and reliable prediction of functional allosteric states, interactions, and mechanisms continue to present highly challenging problems in the field*".[70] Thus, a quantitative characterization via mathematical calculations (rather than simulations), predicting functional allosteric states as a function of chemical denaturant and ligand concentrations, has been presented in the present report. It is expected that these equations will be applied beyond beta-galactosidase, the inhibitors and the chemical denaturants employed in the previous studies.[60][61] On the other hand, the interactions and mechanisms required to switch states, either allosteric (Morpheein model) and or different faces of the same protein (e.g., the Moonlighting concept) as a function of these ligand concentrations in each denaturant concentration have been previously addressed.[60][61]

Another important consequence not mentioned in the last two publications (but gleaned when the equations are perused through[60][61]) is that, despite the fact that the same enzyme was employed in both studies, different interactions and mechanisms emerged, primarily as a function of the different denaturants used, and secondarily as a function of the same galactose concentrations. Thus, despite the same sequence and same assay conditions, the denaturants employed by acting to disrupt the enzyme conformations via different mechanisms served to extend the range of allosteric possibilities. An important lesson here is that sequence conservation does not necessarily serve as a constraint in the manifestation of allosteric possibilities. Rather, the prevailing conditions for unfolding and refolding as regards the potentials for different mechanism(s) of action are the determinant factors to the expression of allosteric states in what has been determined, in this report, to be a quantitative and predictive expression of allosteric states.

Conclusions

In the present report, derivations of thermodynamic equations for the apparent inactivation rate constant,*A*, and product infinity, $[P]_{\infty}$, for the unfolding of β-galactosidase by chemical denaturants (urea and GdnHCl) in the presence of ligand modifiers. These thermodynamic parameters and the ligand concentrations required to affect the migration from one enzyme conformer to the other either by the morpheein allosteric model or via moonlighting dynamics answers a concern

of the community – to quantitatively characterize and predict functional allosteric states. These parameters were derived via calculations rather than by simulations as is prevalent in the field. It is expected that these calculations and their predictive use for allostery will go beyond β-galactosidase, the enzyme employed in these studies, the chemical denaturants – urea and GdnHCl – and galactose as the ligand.

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