Journal of Enzyme Inhibition and Medicinal Chemistry, February 2008; 23(1): 7–15

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Kinetic analysis of urea-inactivation of β-galactosidase in the presence of galactose

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(Received 10 July 2006; accepted 13 April 2007)

Abstract

The effect of galactose on the inactivation of purified β-galactosidase from the black bean, *Kestingiella geocarpa,* in 5 M urea at 50°C and at pH 4.5, was determined.

Lineweaver-Burk plots of initial velocity data in the presence and absence of urea and galactose were used to determine the relevant K_m and V_{max} values, with *p*-nitrophenyl β-D-galactopyranoside (PNPG) as substrate, S. The inactivation data were analysed using the Tsou equation and plots. Plots of $\ln([P]_\infty - [P]_t)$ against time in the presence of urea yielded the inactivation rate constant, *A*. Plots of *A vs* [S] at different galactose concentrations were zero order showing that *A* was independent of [S]. Plots of $[P]_\infty$ *vs* [S] were used to determine the mode of inhibition of the enzyme by galactose, and slopes and intercepts of the $1/[P]_\infty$ *vs*. 1/[S] yielded k_{+0} and k'_{+0}, the microscopic rate constants for the free enzyme and the enzyme-substrate complex, respectively. Plots of k_{+0} and k'_{+0} *vs*. galactose concentrations showed that galactose protected the free enzyme and not the enzyme-substrate complex against urea inactivation via a noncompetitive mechanism at low galactose concentrations and a competitive pattern of inhibition at high galactose concentrations. The implication of the different modes of inhibition in protecting the free enzyme was discussed.

Keywords: *β-galactosidase, urea, inactivation, denaturation, galactose, inhibition, folding intermediates*

Introduction

Over 30 years ago, the Levinthal's paradox was postulated to explain how a protein is preferentially in the native state [1]. It became clear that, although the number of possible conformations available to a polypeptide is so enormous, one conformation generally predominates among the many several conformations [2]. Levinthal concluded that since it is impossible for a polypeptide chain to find its native state by exploring the entire conformational space, a random search mechanism for protein folding could be excluded, and thus there must be some kind of search algorithm in existence for this purpose. This led to the proposal that proteins fold via specific pathways to the native configuration. As protein folding is viewed as a downhill process where the protein explores various conformations on its pathway, the native state is postulated to be the conformation that has about the least or minimal Gibb's free energy (ΔG) amongst other possible conformations. In other words, as viewed from the energy landscape theory or the folding funnel concept [3–7], the native state of a protein is that minimally frustrated heteropolymer with a rugged funnel-like landscape biased towards the native structure [8,9]. The particular conformation taken by the protein influences its biological activity [10,11] and this has influenced the comparison though by limited authors, of the conformation and activity changes during the course of enzyme denaturation [12].

Both physical and chemical agents can be used to achieve the denaturation of proteins. Physical factors include temperature, pH and pressure [10–12]. Chemical factors (solvent denaturation) include high concentrations of chaotropic agents such as urea and guanidine hydrochloride (GuHCl) [2,13,14], certain miscible organic solvents such as alcohol or acetone [10], detergents and surfactants such as sodium dodecyl sulphate (SDS) and dodecyl trimethyl

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ISSN 1475-6366 print/ISSN 1475-6374 online
DOI: 10.1080/14756360701450244

ammonium bromide (DTAB) [15,16]. Since the native conformation of a protein molecule is held together by a large number of bonds, it is therefore conceivable that the disruption of these bonds may require different denaturing conditions with different rates under the same conditions. The disruption of some of these bonds may not be recognizable for some proteins, as the remaining bonds are still sufficient to maintain a structure not very different from the native one. However, the molecule may become more flexible or less compact, with little tertiary structures and more pronounced secondary structures; corresponding to probably what is now being considered as the third state of proteins, the molten globule state [13,17].

Some years ago, a systematic study on the kinetics of the substrate reaction during the irreversible modification of enzyme activity was presented [18]. It has been shown that not only can the apparent inactivation rate for the irreversible modification of enzyme activity be obtained in single experiments, but that the effects of substrate complexing and competition with the modifier can also be ascertained [13,14]. The possibility of partial inactivation by substrate protection leading to the underestimation of rates has to be considered. In the absence of substrate, occurrence of inactivation before significant conformational changes is also a possibility. However, ligands not only increase the rate at which denatured enzymes regain their activity during renaturation in their presence, but also maintain the conformation of proteins during denaturation in their presence. This suggests that ligands act as a folding nucleus about which the remaining constructed regions are easily induced to assume a more biologically active conformation [19] or acts as a stabilizing core for the proteins during denaturation. It was thought that ligands, especially products, inhibitors and substrates increased the stability of enzymes by binding to the native state and thus decreasing the concentration of the unfolded state [20] as in the case of small organic solutes (osmolytes) which aid folding to the native state by raising the chemical potential of the denatured state relative to that of the native state [9,21,22]. Recently, it has been suggested that ligands especially inhibitors can function as molecular chaperones, by not only shifting the conformational equilibrium of its partner (the enzyme) to promote the formation of its active conformation, but also by blocking its active site. On exit of the inhibitor from the active site, the inhibition turns to activation [23].

β-Galactosidase (β-D-galactoside galactohydrolase EC 3.2.1.22) is involved in the depletion of intracellular and cell wall polysaccharides by the preferential hydrolysis of their β-1- and β-1,6- linked galactosyl residues from their non-reducing ends during the early stages of seed germination, and during fruit ripening [24–26]. An important property of galactosidases is their potential to modify blood group specificity of intact human erythrocytes by removing galactose residues from the cell surface glycoproteins [27,28]. The structural conformation of oligosaccharides of human gastric mucin is similar to that of human blood group substances [29]. Most plant β-galactosidases have their pH optima between 2.8 and 4.5 [26,30–32] which are close to stomach and duodenal pH values. This raises the possibility of β-galactosidases (residual in ingested plant foods) attacking gastric mucin. Such an attack would expose the gastric wall to acid and proteolysis. With growing demand for plant milk like say milk, it has become important to study the inactivation of β-galactosidase from plants especially in the presence of galactose, one of the products of its hydrolytic reaction.

Materials and methods

Materials

Fresh, dry, unwrinkled and mature *Kestingiella geocarpa* seeds were bought from the Nsukka main market. *p*-Nitrophenyl β-D-galactopyranoside (PNPG) and urea used were from Sigma Chemical company (St. Louis, MO, USA) and BDH (England) respectively. All other reagents used were of Analar grade.

Germination of seeds

The seeds of *Kestingiella geocarpa* were soaked in distilled water for 48 h to break dormancy. During this period of soaking, the seeds were spread out on a damp jute bag every 6 h for the purpose of aeration. After 48 h, the seeds were transferred to a 0.02%HgI_2 solution for 10 mins for surface sterilization. After washing several times with distilled water, the seeds were decoated, placed on a wet jute bag and allowed to germinate in the dark at 27°C for 96 h. During this period of germination, the seeds were kept moist by wetting the jute bag while being washed with distilled water every 6 h to prevent fungal growth.

Enzyme extraction and purification

The enzyme was extracted and purified according to the method of Chilaka et al [24].

Protein estimation

Protein concentration was determined by the method of Lowry et al [33].

Enzyme assay

Assay for enzyme activity after purification was carried out as described by Chilaka et al [24].

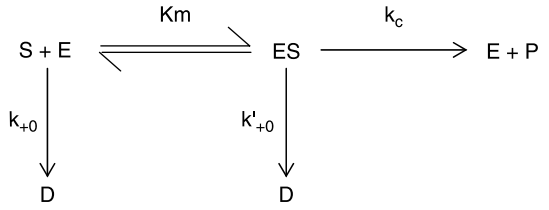
Effect of substrate (PNPG) concentration on urea inactivation of β-galactosidase in the presence and absence of galactose

The enzyme was inactivated by urea in the presence of substrate (PNPG) alone and in the presence of galactose. The assay mixture, in a total volume of 1 mL, contained 0.1 M sodium acetate buffer pH 4.5, different concentrations of the substrate, PNPG (0.025–0.4 mM), in buffer, fixed concentrations of urea (0.0, 4.0, 5.0, 6.0 M urea) and 0.1 mL enzyme. In order to test the effect of galactose on urea inactivation of the enzyme, incubation was now carried out in 5 M urea only, but at fixed concentrations of the substrate, PNPG(0.025–0.4 mM), and varying galactose concentrations, 5.0–50.0 mM. The course of the reaction was monitored by removing aliquots (1 mL) of the reaction mixtures at various time intervals, 0–150 min, and the reaction stopped by the addition of 4 mL of 0.1 M NaOH solution. Absorbance readings were taken at 400 nm using a Pye Unicam SP 8–100 series spectrophotometer and the concentration of *p*-nitrophenol (μmole) released read off from a *p*-nitrophenol standard curve.

Theory

The method for the kinetic analysis of the effects of substrate concentration on urea inactivation of *Kestingiella geocarpa* β-galactosidase in the presence and absence of galactose was a combination of the procedures of Xiao et al [13] and Wang et al [14]. In the presence of substrate, the scheme of enzyme inactivation by denaturants can be written as shown below (Scheme 1):where E, D, K_m, k_c, k_{+0} and k'_{+0} represent the native enzyme, denatured enzyme, the Michaelis constant, the turnover number of the enzyme catalysed reaction in the presence of denaturant, the first order microscopic rate constant for the free enzyme and the first order microscopic rate constant for the enzyme-substrate complex, respectively. All the kinetic constants are functions of the denaturant concentrations and thus functions of the apparent inactivation rate constant, A [2,13,14]. During enzyme inactivation, if the substrate concentration is considered constant during the period of observation, the rate of enzyme inactivation is given by

$$\delta[E]/\delta t = A[E_t] \tag{1}$$



Scheme 1.

where

$$A = \frac{k_{+0}K_m + k'_{+0}[S]_0}{K_m + [S]_0} \tag{2}$$

is the apparent inactivation rate constant. Integrating (1) with boundary condition

$$t = 0,\ [E_t] = [E]_0,$$

then

$$[E_t] = [E]_0 e^{-At} \tag{3}$$

Introducing Equation (3) into Equation (1) yields

$$\delta[P]/\delta t = v_0 e^{-At} \tag{4}$$

where

$$v_0 = k_c[E]_0 \tag{5}$$

is the initial velocity of the native enzyme catalysed reaction in the presence of denaturant. With boundary conditions t = 0, [P] = 0, Equation (4) integrates to

$$[P] = v_0/t(1 - e^{-At}) - [P]_\infty(1 - e^{-At}) \tag{6}$$

where

$$[P]_\infty = v_0/A = \frac{K_c[E]_0[S]_0}{k_{+0}K_m + k_{+0}[S]_0} \tag{7}$$

and $[P]_\infty$ is the concentration of product when the reaction time is sufficiently long. Only taking the reciprocal of Equation (7), we have

$$1/[P]_\infty = \frac{k_{+0}K_m}{K_c[E]_0[S]_0} + \frac{k'_{+0}[S]_0}{K_c[E]_0[S]_0} \tag{8}$$

$$= \frac{k_{+0}K_m}{V_{max}[S]} + \frac{k'_{+0}}{V_{max}} \tag{9}$$

Transforming Equation (9) into a straight line equation of the form, y = mx + c, then

$$1/[P]_\infty = \frac{k'_{+0}}{V_{max}} + \frac{k_{+0}K_m}{V_{max}}\frac{1}{[S]} \tag{10}$$

From these equations, a plot of $1/[P]_\infty$ vs 1/[S] will yield k_{+0} and k'_{+0} from the intercepts and slopes respectively.

Results

A Lineweaver-Burk plot of initial velocity data of the native enzyme in the absence of urea gave a K_m of 0.25 mM and a V_{max} of 10.0 μmole/min and galactose was a competitive inhibitor with a K_i of 26.0 mM.

Time curves (plots of $[P]_t$ (*p*-nitrophenol released) *vs* time, t) for 0, 4, 5 and 6 M urea showed that the concentration of product [P]([pNP]) formed at any

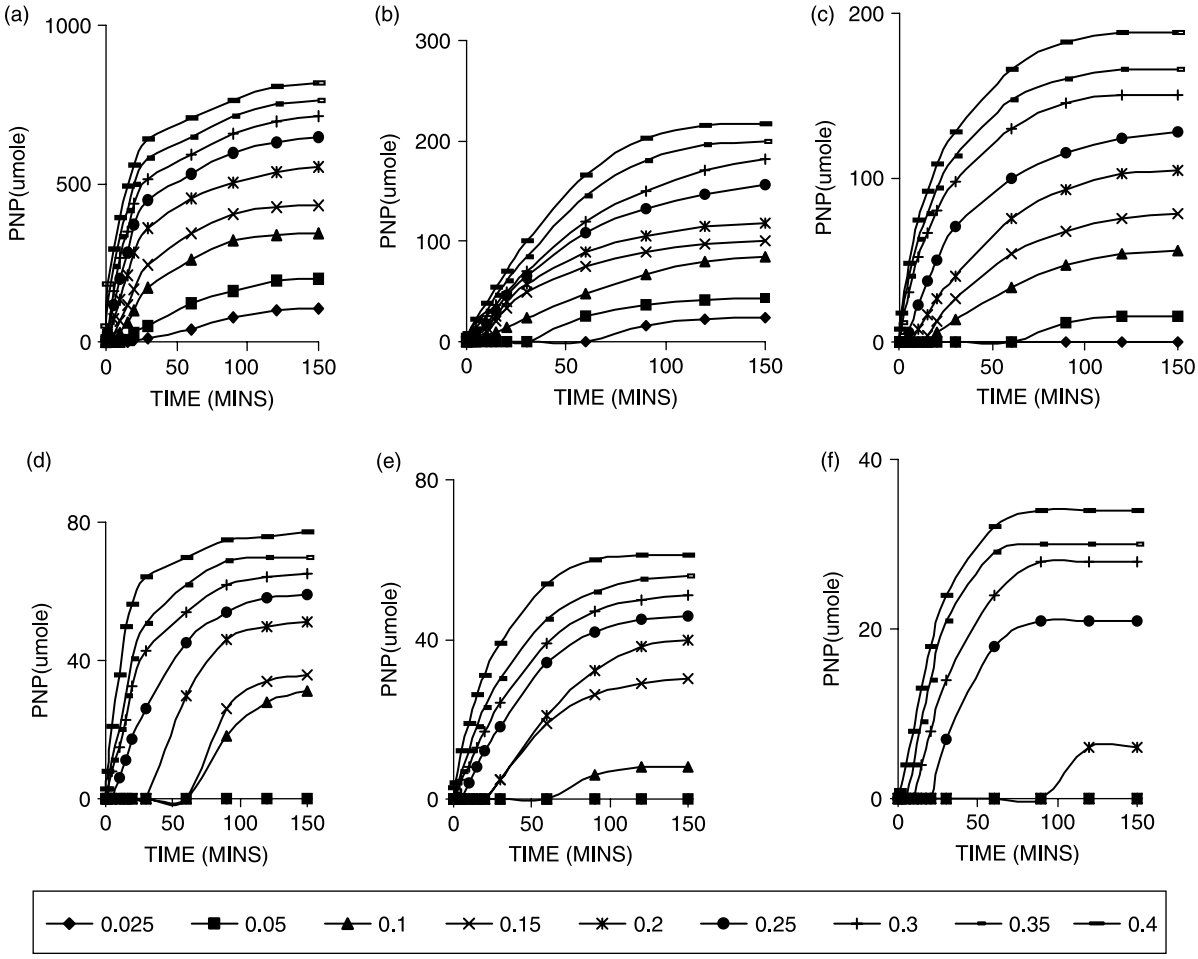


Figure 1. Kinetics of the inactivation of β-galactosidase in the absence and presence of 5 M urea at 50°C,pH 4.5, and at different concentrations of substrate, PNPG, [0.025–0.40 mM] **a:** In the absence of urea (No urea); **b:** in the presence of 5 M urea; **c:** in the presence of 5 M urea and 5 mM galactose; **d:** in the presence of 5 M urea and 10 mM galactose; **e:** in the presence of 5 M urea and 20 mM galactose; **f:** in the presence of 5 M urea and 50 mM galactose.

time interval t, was directly related to the substrate concentration ([pNPG]) (Figures 1a and 1b). With increase in reaction time t, $[P]_t$ approached a constant value $[P]_\infty$, at each pNPG concentration. It was observed that the $[P]_\infty$ for each $[S]_0$ decreased with corresponding increase in urea concentration (Figures 1a and 1b). Further decreases of $[P]_\infty$, were observed in the presence of galactose, and with increase in galactose concentration (Figures 1c–1f). In the presence of urea, a lag phase was encountered, especially at low pNPG concentrations before pNP formation commenced, which became more pronounced at higher galactose concentrations. It was observed that the K_m increased, slightly with increasing urea, and largely with galactose concentrations, 0.5–28.2 mM (Figure 2) while the V_{max} correspondingly decreased. The slight increase in K_m with increasing urea concentrations shows that the urea decreased the affinity of the enzyme for its substrate, pNPG. This effect is reflected in a decrease in the $[P]_\infty$ for corresponding increases in the urea concentration. Plots of $\ln([P]_\infty - [P]_t)$ *vs*. time t, (Figures 3a and 3b) gave straight lines (first order kinetics) with slopes corresponding to *A*, the apparent inactivation rate constant. Plots of A *vs* [S], in the presence and absence of galactose gave zero order (Figures 4ai and 4aii),

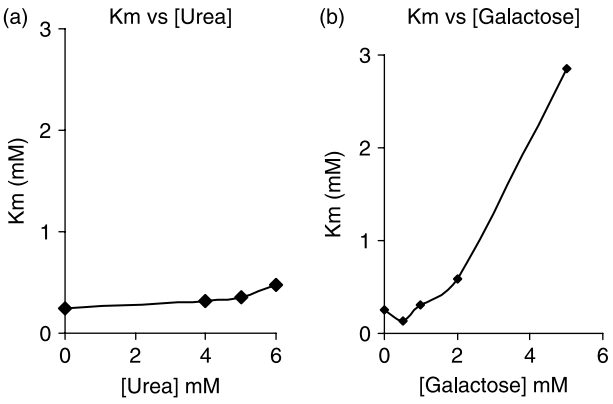


Figure 2. Effect of urea and galactose on the K_m of β-Galactosidase using pNPG as substrate. **a:** urea only **b:** in the presence of 5 M urea and galactose. K_m was calculated from Lineweaver-Burk plots of initial velocity data at the concentrations of urea and galactose indicated.

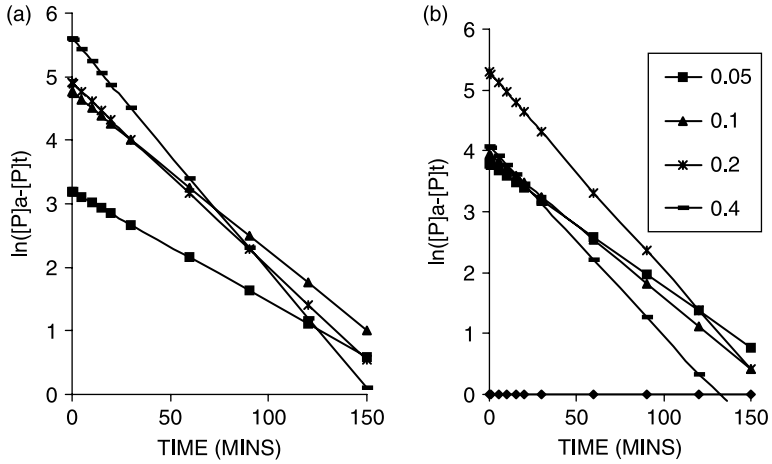


Figure 3. Semilogarithmic plot of P(μM) *vs* time (t) of data in Figure 1. a: ln([P]a-[P]t) *vs* t for 5 M Urea. b: ln([P]a-[P]t) *vs* t for 5 M Urea,10 mMGalactose. N.B: [P]a = [pNP]; [P]t = [pNP]t.

showing that the substrate has no protective effect on the enzyme inactivation and thus A will be independent of substrate; that is $A = k_{+0} = k'_{+0}$, where k_{+0} and k'_{+0} are the microscopic rate constants for the free enzyme and the enzyme-substrate complex respectively. Furthermore, there was an increase in A with increase in galactose concentration up to 10 mM, while a further increase in galactose concentration above 10 mM caused a decrease in A (Figure 4b). It has been argued that the decrease in enzyme activity is by reversible binding of denaturant. Values of A were $0.0135–0.0380 s^{-1}$ for β-galactosidase, which compares favourably with a value of $0.016 s^{-1}$ for papain; thereby making inhibition by urea unlikely [13]. Experimentally, the type of inhibition can be ascertained by studying the effect of [S] either on the apparent rate constant, A or on $[P]_\infty$ [18]. From the effect of [S] on A, noncompetitive inhibition is involved when A is independent of [S],while for competitive or uncompetitive inhibition, the plot of $1/A$ against [S] or 1/[S] will be a straight line respectively. Alternatively, from the effect of [S] on $[P]_\infty$, a competitive inhibition is predicted when a plot of $[P]_\infty$ against [S] gives a straight line passing through the origin. For noncompetitive inhibition, the plot of $1/[P]_\infty$ against 1/[S] will be a straight line whereas for uncompetitive inhibition $[P]_\infty$ will be independent of [S]. Plots of $[P]_\infty$ vs. [S] (Figure 5) gave straight lines passing through the origin for higher concentrations of galactose, indicating competitive inhibition, while at lower concentrations the intercept is at the $[P]_\infty$ axis, indicating noncompetitive inhibition.

Plots of $1/[P]_\infty$ *vs.* $1/[S]_0$ at different galactose concentrations yielded k_{+0} and k'_{+0}. Plots of k_{+0} and k'_{+0} *vs.* galactose concentrations (Figure 6) showed that k_{+0} *vs.* [galactose] was a hyperbolic curve with k_{+0} decreasing from 0.0724 to $0.0198 s^{-1}$, while k'_{+0} gave a hyperbolic curve with k'_{+0} increasing from 0.0072 to $0.0545 s^{-1}$. This demonstrated a protection of the free enzyme and not the enzyme-substrate complex by the galactose.

Discussion

It has been reported that during chemical or physical denaturation of many enzymes, inactivation may or may not parallel overall conformational changes [13]. It has been argued that during measurements of conformational changes, no substrate was present as compared to activity measurements [14]. This

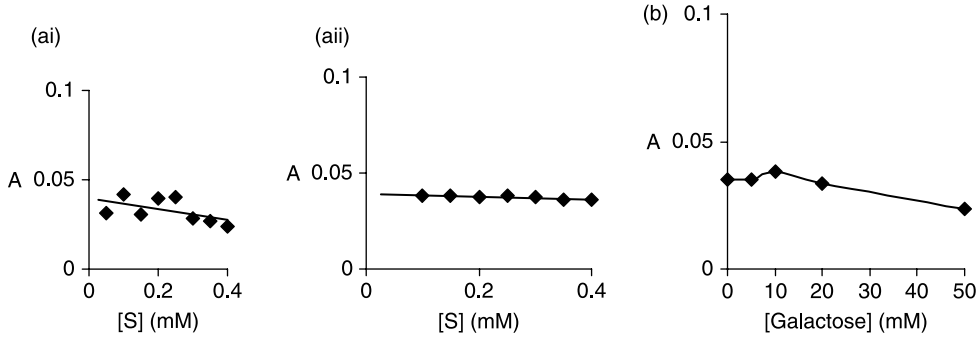


Figure 4. Plot of apparent inactivation rate constant A against substrate (pNPG) concentration. **ai:** In the presence of 5 M urea only; **aii:** In the presence of 5 M Urea and10 mM galactose.(b) A *vs* [galactose].

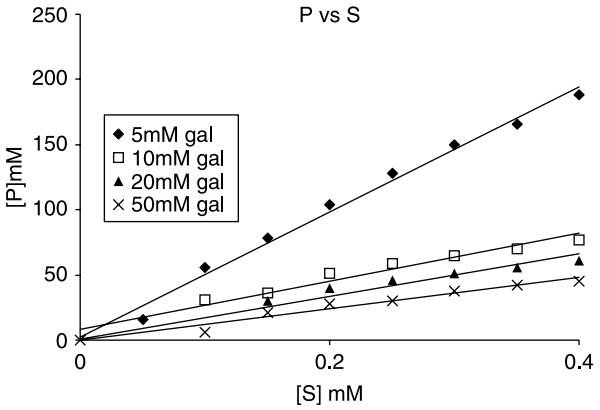


Figure 5. Plots of $[P]_\infty$ against [S] for β-galactosidase in the presence of 5 M urea and **a:** 5 mM galactose **b:** 10 mM galactose **c:** 20 mM galactose **d:** 50 mM galactose.

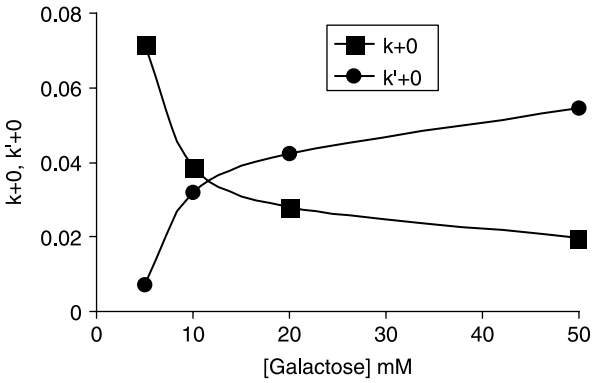
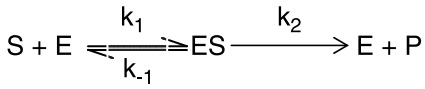


Figure 6. Plot of $k + 0$, $k' + 0$ *vs* [Galactose]. k_{+0} and k'_{+0} being the microscopic inactivation rate constant for the free enzyme and enzyme-substrate complex respectively.

raises the question of substrate protection or partial reactivation leading to underestimation of the rates or extents of inactivation. Recently, a systematic study of the kinetics of the substrate reaction during irreversible modification of enzyme activity was carried out. Both the apparent rate constant for irreversible modification of enzyme activity and effect of substrate complexing and competition with the modifier were determined in a single experiment [13,14,18]. This approach has been extended to the study of the inactivation kinetics of β-galactosidase by urea in the presence of galactose.

A lag phase was observed in the presence of urea, which became more prominent on addition of galactose. Urea, or galactose, separately, can decrease the activity of the enzyme. The further increase in lag phase with increase in galactose concentration would arise from inactivator/inhibitor synergism, with the increase proportional to the sum of the concentrations of urea and galactose. Lag phases usually describe hysteresis, a phenomenon illustrating the concept of "enzyme memory", which involves changes of an enzyme from one active/catalytic state to another. A hysteretic enzyme usually responds slowly to a rapid change in the concentration of ligand, substrate or modifier [34]. The time of conversion from one kinetic form to another, in the presence of the ligand, is slow relative to the rate of the over-all catalytic reaction (a contrast to rapid equilibrium kinetic mechanisms). The slow nature of these responses comes from a rearrangement of the building blocks of the enzymes, the secondary structures [35]. In other words, in the presence of urea alone, the enzyme tertiary structure breaks down to basically a structure populated more by secondary structures, which become the building blocks for a new enzyme conformation stabilized by the ligand in the medium. The supposedly third phase of proteins, the molten globules, is populated mainly by secondary structures and remnants of tertiary interactions. Moreover, extremes in conditions such as pH, ionic strength, temperature and denaturants would readily induce the equilibrium molten globule state in a protein [36,37,38,39]. Kinetic intermediates have been identified on the folding pathway of many proteins [40,41,42,43] in native-like conditions [39]. In the presence of the ligand, the effect of the urea might be to induce some unstructuredness in the enzyme molecule. By the interplay of reversible inhibitory mechanisms with the ligand bound to the disordered protein molecule, the enzyme molecule experiences a conformational excursion around an energy minimum topologically favoring the native state. In β-galactosidase, as galactose concentration increases, more galactose binds to the inactive or denatured enzyme and renaturation of the enzyme is enhanced so that there would be more free enzyme to combine with the substrate per unit time, thereby lowering the concentration of enzyme inactivated per unit time and increasing the value of the partition ratio, $r = [P]_\infty/[E]_0$. This induces hysteresis. Hysteretic enzymes are usually involved in metabolic regulation. For example, β-galactosidase is involved in metabolic regulation during seed germination and fruit ripening [26,27,30].

There was a slight increase in K_m in the presence of urea only, when compared to 5 M urea and galactose concentrations $\leq$20 mM. However in 50 mM galactose, it becomes clear that the predominant effect on the enzyme is inhibition by galactose with little or no perturbation of the enzyme active site by the urea. This becomes even more apparent in the plot of the apparent inactivation rate constant, A against galactose concentrations. From the graph, there was very little change in A for 5 M urea only (no galactose) when compared to 5 mM galactose. Although there was a slight increase in A up to 10 mM galactose as compared to the absence of galactose, there was a drop in the value of A for 20 mM galactose and a large decrease in the value of A for 50 mM galactose.This shows that, at this concentration,



Scheme 2.

competitive inhibition was dominant while perturbation of the enzyme active site by the 5 M urea was negligible. The slight increase in K_m with increasing urea concentrations would therefore indicate that the urea caused a minor decrease in the affinity of the enzyme for its substrate. This effect is reflected in a decrease in the $[P]_\infty$ for corresponding increases in the urea concentration. Employing the steady state conditions as follows (Scheme 2):

$$[S] >> [ES], [P] \tag{11}$$

$$K_m = (k_{-1} + k_2)/k_1 \tag{12}$$

as k_2 is a component of K_m, chemical/catalytic events contribute to changes in K_m. In agreement with our earlier suggestion, the effect of urea might be to induce some unstructuredness in the enzyme molecule resulting in the disorganization of the essential residues at the catalytic site. This disorganization does not adversely affect the binding of galactose in the galactose binding site at the active site region. In other words, urea inactivates the enzyme by interfering with the catalytic step, perhaps due to a limited protein unfolding in the active site region without necessarily inducing global conformational changes. Also, many authors have shown that inactivation occurs before measurable conformational changes during enzyme denaturation [42].

During the inactivation of *Kestingeilla geocarpa* β-galactosidase by urea, the substrate, pNPG, offers no protection to the enzyme against urea inactivation. However, galactose not only protects the enzyme from urea inactivation by binding to the free enzyme, but also does that by using different inhibition patterns. A Lineweaver-Burk plot of initial velocity data of the native enzyme in the absence of urea showed that galactose was a competitive inhibitor. In the presence of urea, galactose exhibited noncompetitive inhibition pattern at lower concentrations of galactose ($\geqq$ 5 mM), while at higher concentrations ($>$ 20 mM), it reverted to its conventional competitive inhibition pattern. In an earlier work, we reported a change in mode of inhibition for glucose with β-galactosidase from competitive inhibition pattern to uncompetitive as glucose concentration increased [24]. A ligand induced isomerization (34) of the enzyme could occur either via a competitive, noncompetitive or an uncompetitive pathway. In the absence of galactose, the unstructuredness in the enzyme molecule induced by urea enables the binding region in the denatured form to become "binding non-competent" with regards to the substrate. On the ligand leaving the active site, inhibition turns to activation. In the denatured state, the protein may exhibit moonlighting properties peculiar to intrinsically unstructured proteins (IUPs) while the ligand, galactose, acts as a chaperone. Structural reorganization around the partner (the enzyme) is one of the three principal, non-exclusive mechanisms [23] employed by moonlighting proteins to switch functions. Moonlighting proteins are able to fulfill more than one, apparently unrelated function [44–46]. In this case, the enzyme acts as a receptor to its ligand, which binds in a competitive manner while at the same time using the same site (its active site) for enzymatic purpose. Another interesting aspect of this enzyme with respect to the suggested moonlighting property is its ability to bind to a partner molecule in different conformations. This is the second of the three principles (non-exclusive mechanism) employed by moonlighting proteins to acquire the native conformation [23]. In this case, the enzyme has another site different from the catalytic site in which the ligand binds in a noncompetitive manner, still eliciting reformation of either the whole conformation or solely that of the active site, even though with an increase in the apparent inactivation rate constant, *A*. This could be understood from the point of view that the enzyme form involved in noncompetitive inhibition would be different from the enzyme form for competitive inhibition; which would still be different from the original enzyme form. Important also, is that the different conformers do not have the same minima of energy since their K_mS and V_{max}S vary. Thus, the native conformation of a protein is marginally stable, and may not necessarily be the most stable conformation [24]. The minima in the rugged energy landscape of the folding funnel [4] applied to our enzyme may depict various conformations stabilized by galactose. The interconversion amongst the various conformations is a function of the concentration of the galactose. However, the magnitude of the energy barriers between different related conformers can easily be lowered by the various denaturants [47], in this case urea. While urea lowers the energy barriers, the various galactose concentrations stabilize the different conformers being renatured.

In summary, in the presence of urea alone, the enzyme tertiary structure might break down to basically a structure populated more by secondary structures and very little tertiary interactions, the molten globule. However, in the presence of the ligand, the effect of the urea might only be to induce some unstructuredness in the enzyme molecule in the active site region In this case, urea inactivates the enzyme by interfering with the catalytic step, perhaps due to limited protein unfolding in the active site region without necessarily inducing global conformational changes. Inactivation, thus, occurs

before measurable conformational changes during enzyme denaturation. In the denatured state, the protein may exhibit moonlighting properties peculiar to intrinsically unstructured proteins (IUPs) while the ligand, galactose, acts as a chaperone. The minima in an rugged energy landscape of the folding funnel [4] applied to our enzyme may depict various conformations stabilized by galactose.

In conclusion, galactose stabilization of *K. geocarpa* β-galactosidase against urea denaturation implies that the dominant inactivation/denaturation pathway involves changes in the enzyme active site. Also, denaturing the β-galactosidase by urea in the presence of galactose, may not necessarily inactivate the enzyme because of the protection offered by galactose, a product of its hydrolytic reaction

Finally, this work poses a very important question: how denatured would a denatured state be in the presence of a stabilizing ligand?.

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