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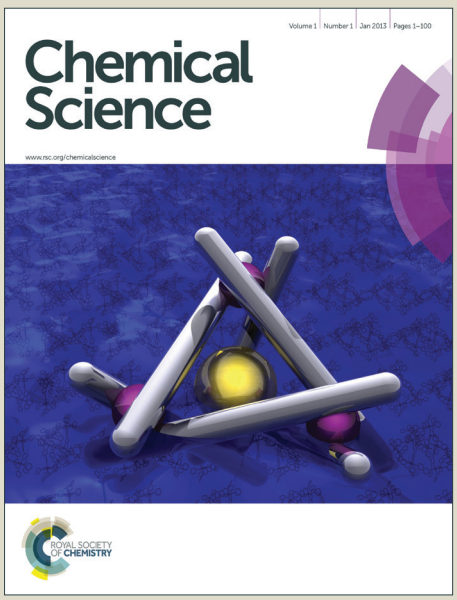

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EDGE ARTICLE

Elucidating the Relationship between Substrate and Inhibitor Binding to the Active Sites of tetrameric β-Galactosidase

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The activities of hundreds of single molecules of β-galactosidase were monitored in the presence of fluorogenic substrates and two strong binding inhibitors—D-galactal and N-p-bromobenzylamino-hydroxymethyl-cyclopentanetriol (NpBHC). The stochastic binding and release of the inhibitors to single β-galactosidase molecules was studied in both pre-steady state and steady state conditions. The effect of inhibition on enzyme activity is described and compared for both inhibitors. The inhibitor exchange rate and the substrate turnover rate were computed for individual enzyme molecules. These parameters are shown to be heterogeneous across the enzyme population. We demonstrate an inverse correlation between these parameters thus demonstrating that competitive inhibition is tightly coupled to the nature of the active site of individual enzyme molecules.

Introduction:

Competitive enzyme inhibition is a fundamental mechanism in biochemistry. Bulk kinetic analyses, however, only give a limited picture of enzyme reaction mechanisms, as they assume the same behaviour for all enzyme molecules in a population. Single molecule studies of enzymes provide a high resolution picture of the underlying behaviour of individual molecules that gives rise to their bulk properties. While bulk studies only provide ensemble averaged properties, single molecule studies shed light on the inherent heterogeneity between different molecules[1-7] and within one molecule over time[3, 8-15]. Such studies also enable the detection of transient kinetic and dynamic events[16-23] that cannot be detected in bulk systems.

In this paper, the competition between inhibitor and substrate molecules for the active sites of hundreds of individual enzyme molecules is studied in arrays of femtoliter-sized wells etched into the end of an optical fibre bundle[5, 7, 21]. Specifically, we observe and compare stochastic binding and release of two inhibitors, D-galactal and NpBHC, to the tetrameric enzyme β-galactosidase to determine if the inhibitors bind or release in a sequential manner. We compare the enzyme activity before and after inhibitor binding and release to investigate if inhibitor binding changes the enzyme conformation. Finally, using autocorrelation analysis the inhibitor exchange rates of the enzymes are correlated to the substrate turnover rates.

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The inhibition of β-galactosidase by D-galactal has been extensively studied both in bulk and at the single molecule level[21, 24-26]. D-galactal binds to β-galactosidase to form a glycosyl-enzyme complex, which dissociates slowly to regenerate the free enzyme and release the product 2-deoxygalactose, with an off-rate of ~$2x10^{-3}$ s^{-1} [21, 24]. The inhibition has characteristic slow binding kinetics with a reported on-rate constant of ~$4x10^{2}$ $M^{-1}s^{-1}$ [24]. Using an optical fibre bundle set up it was demonstrated previously that the release of D-galactal from the tetrameric enzyme β-galactosidase occurs in a single step[21]. It was found that the subunits of β-galactosidase release D-galactal in a cooperative manner. In addition, the binding and release of D-galactal was demonstrated to induce a conformational change of the enzyme resulting in a change in the enzyme activity. Therefore, the enzyme does not always revert to the activity it had before inhibitor binding. The activity profile of the enzyme in the presence of inhibitor thus exhibits a fluctuation between distinct activity levels.

NpBHC is another potent inhibitor of β-galactosidase modelled after mannostatin, which is a strong inhibitor of mannosidase[27]. It is a reversible transition state inhibitor with an inhibition constant of 0.5 nM (calculated from bulk experiments). Bulk studies show that NpBHC is a tight binding inhibitor with an on-rate constant of $4.5x10^{5}$ $M^{-1}s^{-1}$ and an off-rate of $2x10^{-3}$ s^{-1}, estimated by fitting the steady state progress curves to the integrated equation described in[28]. Therefore, under steady state conditions, NpBHC has a low dissociation and association rate on the order of 10^{-3} s^{-1}, which makes it ideal for observing the binding and release from β-galactosidase because inhibitor binding events can be readily distinguished from substrate turnover events. In this paper, we

study the single molecule inhibition of β-galactosidase by NpBHC and compare it to inhibition with D-galactal.

Experimental

Materials: β-galactosidase from E. coli (grade VIII) was purchased from Sigma–Aldrich and dissolved and diluted in 1X PBS/1 mM MgCl2 buffer (pH=7.4). The enzyme was purified using HPLC and characterized on a native gel (SI 1). Stock solutions of 100 mM D-galactal (1,5-anhydro-2-deoxy-d-lyxo-hex-1-enitol) (Sigma–Aldrich) in PBS/$MgCl_2$, 50 mM N-p-bromobenzylamino-hydroxymethyl cyclopentanetriol (NpBHC) in PBS/$MgCl_2$, 100 mM resorufin-β-D-galactopyranoside (RDG) (Invitrogen, Carlsbad, CA) in DMSO, 100 mM resorufin sodium salt (Invitrogen) in DMSO, 10 mM fluorescein di-β-D-galactopyranoside (Invitrogen) in DMSO and 100 mM fluorescein (Invitrogen) in DMSO were aliquotted and stored at −20°C. Further dilutions of all the substrates and inhibitors were made in reaction buffer containing 1X PBS, 1 mM $MgCl_2$, 0.005% w/v Tween-20 and 0.005% w/v BSA, pH =7.4. Optical fibre bundles (Ø=2mm) containing 50,000 optical fibres (Ø=4.5μm) were purchased from Schott (Southbridge, MA).

Microchamber array fabrication and single molecule experiments: High density optical fibre microarrays were employed to isolate single enzyme enzyme molecules. Microcamber array fabrication and isolation of single enzyme molecules are described elsewhere[5, 21].

Pre-steady state inhibition experiments: Pre-steady state experiments were used to investigate whether the inhibitor release from the tetrameric enzyme happens in a cooperative or sequential manner. 1 μL of a pre-incubated mixture of enzyme and inhibitor (3.6 nM of enzyme and 100 μM of D-galactal incubated for 15 minutes or 3.6 nM of enzyme and 10 nM of NpBHC incubated for 20 minutes) was diluted into 1 mL of 100 μM RDG solution to give a final enzyme concentration of 3.6 pM and inhibitor concentration at least an order of magnitude lower than K_i. After the final dilution, the reaction mixture was immediately sealed into optical fibre wells and time-lapse imaging was performed.

Steady state inhibition experiments: Steady state experiments were used to compare the activities of the individual enzyme molecules before and after inhibitor binding. For D-galactal experiments, 1 μL of 3.6 nM enzyme was diluted into the 1 mL solution containing 100μM of RDG and 20 μM of D-galactal. For NpBHC experiments, 1 μL of 3.6 nM enzyme was diluted into the 1 mL solution containing 100μM of RDG and 0.5 nM of NpBHC). These inhibitor concentrations were chosen because they are equal to the inhibition constant (K_i) of the inhibitor. After the final dilution, the reaction mixture was immediately sealed into the optical fibre wells and time-lapse imaging was recorded.

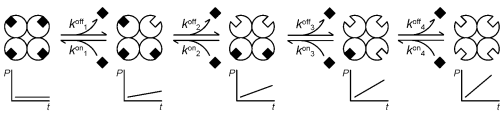
Multiple substrate reactions: Multiple substrate reactions were conducted by diluting 1 μL of 3.6 nM enzyme solution into 1 mL of a solution containing 10 μM each of both RDG and FDG. After the final dilution, the reaction mixture was immediately sealed into the optical fibre wells and time lapse imaging was recorded every 30 s for at least 20 min with an exposure time of 2 s for both fluorophores. The filter wheel was set to alternate between the resorufin and fluorescein cubes for each measurement.

Imaging: Time lapse images were recorded for at least 30 minutes with an upright Olympus (Tokyo, Japan) BX61 microscope equipped with a short arc mercury lamp (Ushio, Tokyo, Japan) and a CCD camera (Sensicam QE; Cooke Optics, Romulus, MI). A filter set with λ_{ex} = 571 nm and λ_{em} = 584 nm (Chroma Technology, Rockingham, VT) was used for the resorufin product, and a filter set with λ_{ex} = 475 nm and λ_{em} = 540 nm was used for the product fluorescein (Chroma Technology, Rockingham, VT). Images were recorded every 15 s or 30 s using an exposure time of 2 s under reduced excitation light (ND = 1). The total reaction time lasted for at least 15 minutes.

Results and discussion:

First, in analogy to the single molecule studies on inhibition by D-galactal[21], the inhibition of β-galactosidase by NpBHC was investigated to determine if β-galactosidase subunits release NpBHC in an independent or cooperative manner. In a pre-steady state experiment, all active sites of the enzyme are first blocked by pre-incubating with the inhibitor. The enzyme-inhibitor complex is then diluted into a substrate solution such that the final concentration of the inhibitor is orders of magnitude lower than the inhibition constant K_i. Under these pre-steady state conditions, re-binding of the inhibitor to the enzyme is highly unlikely and therefore release of the inhibitor from the enzyme subunits can be exclusively monitored by measuring the onset of substrate turnover. If NpBHC release from the subunits is cooperative, the enzyme is expected to jump from a state of no activity to its highest activity state without any intermediate activity levels. On the other hand, if the subunits release NpBHC independent from each other, intermediate activity levels are expected before the enzyme reaches its maximum activity (Scheme 1).

We conducted pre-steady state experiments where β-galactosidase is pre-incubated with NpBHC for 15 minutes and then diluted 1000 fold into substrate solution. The results are shown in Figure 1. Inhibitor dissociation is indicated by the onset of an increase in fluorescence as shown in Figure 1a. A histogram of the off-times (see SI 3 for a description of how the off-times were determined) before inhibitor release shows an exponential decay (Figure 1b), with a dissociation rate of $2.8\text{x}10^{-3} \pm 0.8\text{x}10^{-3}$ s^{-1}, which is consistent with the dissociation rate computed from bulk experiments ($2.6\text{x}10^{-3}$ s^{-1}, SI 2). Notably, the dissociation rate of NpBHC is also similar to the dissociation rate of D-galactal[21].



Scheme 1. Schematic showing model for sequential inhibitor release from a tetrameric enzyme[21]. In this model, the inhibitors are released from the tetrameric enzyme in a sequential manner, and the enzyme can exist in five different states as shown, depending on the number of active sites blocked by the inhibitor. Each of the five forms have distinct activities as depicted by the product (P) vs time (t) profiles under the respective enzyme state. For example, the enzyme state that has all the active sites free will have higher activity than the enzyme that has only three free active sites and one blocked active site and so on. To go from the state of no activity (i.e. all active sites blocked) to the state of highest activity (i.e. all active sites free), the enzyme has to go through all the intermediate states.

To investigate whether the inhibitor is released in a cooperative or sequential manner, the fluorescence intensity trajectories in Figure 1a are converted to substrate turnover rates by taking the derivative of the trajectories and multiplying by a calibration constant followed by correction for photobleaching[21]. Turnover rates of a few representative trajectories from the pre-steady state experiment are shown in Figure 1c. The turnover rate increases in a stepwise manner for many enzyme trajectories. That is, multiple levels of activity are observed after inhibitor release before maximum activity is reached for many enzyme trajectories. Since the final concentration of NpBHC is orders of magnitude lower than the inhibition constant, it is highly unlikely that NpBHC rebinds β-galactosidase during the observation time. The different levels of activity therefore may be attributed to sequential inhibitor release from the enzyme. This behaviour is unlike that of D-galactal where the enzymes were observed to go from a state of no activity to the highest activity level in a single step[21].

In Figure 1c, an inhibitor release event, characterized by a sudden change in turnover rate, can be clearly distinguished from the background variation in the turnover rate. The turnover rate between two subsequent inhibitor release events is stable and is defined as an 'activity level'. Since β-galactosidase is a tetramer, each enzyme molecule is expected to display 5 different activity levels (including the inactive state) corresponding to the stoichiometry of inhibitor binding, before it reaches maximum activity (Scheme 1). While several enzymes show different levels of activity, demonstrating sequential inhibitor release, the number of activity levels observed is not the same for all enzymes as can be seen in Figure 1c. The number of activity levels for each trajectory was determined by using a method based on the student t-test[29] (SI 3). The number of activity levels observed ranged from 2-4, unlike the expected five levels of activity for each enzyme based on scheme 1.

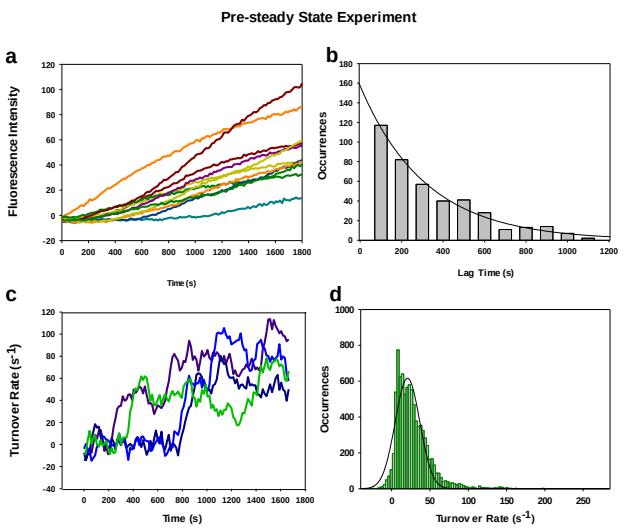


Figure 1. (a) Pre-steady state inhibition experiment with the inhibitor NpBHC performed by incubating 3.6 nM of β-galactosidase with 10 nM inhibitor followed by a 1000-fold dilution into the substrate RGD (100 μM) and sealing in optical fibre wells. The figure shows background-corrected and smoothed (median filter = 1) fluorescence time traces obtained by imaging the fibre. Initially all the active sites of the tetrameric enzyme molecule are bound by the inhibitor resulting in no build up of fluorescence. After the inhibitor is released from the enzyme, the fluorescence intensity increases due to the enzyme regaining activity. Different lag times can be observed in the trajectories for different molecules due to in the stochastic nature of the dissociation times[21]. In the absence of inhibitor no such lag time is observed[21] (b) Typical histogram of the lag times from a pre-steady state experiment with NpBHC used to determine the k_{off}. (c) Turnover rates calculated for a few trajectories in (a). The slopes at every point in the fluorescence intensity trajectories were computed to give the change in fluorescence intensity per unit time. The slopes were then converted into numbers of product molecules per unit time using a calibration curve obtained by sealing various concentrations of resorufin into optical fibre wells and recording the raw fluorescence intensity value. (d). Histogram of average turnover rates calculated for each activity level distinguishable from traces in (c).

The average turnover for each activity level observed in Figure 1c was computed (SI 3). A histogram of these turnovers is shown in Figure 1d along with the best fit obtained for a normal distribution. The turnover distribution does not fit well to a normal distribution. Multiple peaks can be identified in the histogram in Figure 1d and may be generated from multiple populations of the enzyme, rather than a single population of enzyme. These multiple populations of the enzyme could be a result of the number of active sites blocked by the inhibitor. For example, the population of enzyme with all active sites blocked has zero activity; the population of enzyme with three of the active sites blocked has the lowest level of activity and so on. If the activity of each enzyme state in scheme 1 is well quantized, then the histogram in Figure 1d is expected to contain five peaks. However, due to broad static heterogeneity of the enzyme activity, the five peaks are not clearly distinguishable and it is not possible to quantize the activity of the five different states.

The conclusion for these results indicates that D-galactal and NpBHC differ in the kinetics of inhibitor release from β-galactosidase subunits. While D-galactal release seems to be strictly cooperative, NpBHC seems to be released sequentially from the four enzyme subunits. Sequential release of inhibitor was reported by Craig et al.[26] in the case of D-galactal for a minor fraction of the total molecules observed. In our studies, we were only able to observe cooperative release for D-galactal both in this study and in Reference 21. In contrast, for NpBHC, we observe sequential release for approximately 30% of the enzyme molecules that show an initial lag in activity.

The variations in enzyme turnover rates due to inhibitor binding and release can also be monitored under steady state conditions ([I] = K_i). Beginning with an un-inhibited free enzyme, we can observe fluctuations in enzyme activity. Steady state experiments of NpBHC and D-galactal were performed at an inhibitor concentration equal to the inhibition constants (K_i = 0.5 nM for NpBHC and K_i = 20 μM for D-galactal). The steady state trajectories are shown in Figure 2a. During the initial stages of the trajectories, the enzymes are active resulting in an increase in fluorescence. A pause in fluorescence build up indicates the duration during which the inhibitor is bound to the enzyme. Figure 2b shows the turnover rate variations during a steady state experiment with NpBHC and D-galactal respectively.

Significant fluctuations in the enzyme turnover can be observed in the presence of NpBHC as well as D-galactal. The enzyme fluctuates between distinct activity states, indicating an inhibitor binding or release event. A histogram of the turnover rates of the enzyme in the presence of the inhibitors is shown in

Figure 2c (only the durations in which the enzyme was active were considered). While the activity of the enzyme is lower in the presence of both inhibitors, the activities are lower for D-galactal. Even though NpBHC is a more potent inhibitor of β-galactosidase, D-galactal has a stronger effect on the enzyme activity. D-galactal is a reversible inhibitor where the conversion of β-galactosidase from an inactive to an active state is accompanied by conversion of D-galactal to 2-deoxygalactose[24] whereas NpBHC is a classical reversible inhibitor, where the release is accompanied by the recovery of both the enzyme and inhibitor. Possibly, since the enzymatic reaction of β-galactosidase with D-galactal proceeds through the transition state to the production of the product, the enzyme conformation is likely significantly altered[25], resulting in lower activities.

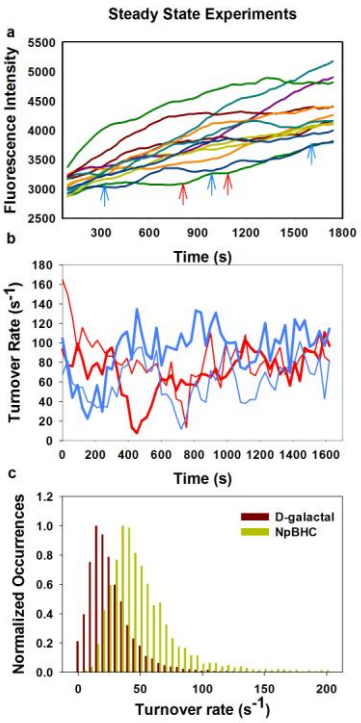


Figure 2. (a) Representative trajectories of raw fluorescence intensity in the active wells under steady state reaction conditions. NpBHC binding and release events are indicated for one representative trajectory (green). The blue arrows indicate when inhibitor binds to the active site and resulting in lack of substrate turnover and a stable fluorescent intensity, while the red arrows indicate a later time point when the inhibitor is released from the active site resulting in substrate turnover and an increase in fluorescence intensity. (b) An example of substrate turnover rates calculated from fluorescence intensity trajectories for D-galactal (blue traces) and NpBHC (red traces). Distinct levels of activity within the duration of the experiment can be identified indicating different stochiometries of binding of the inhibitor to the subunits of the tetrameric β-galactosidase. (c) Histogram of the average turnover rates under steady state reaction conditions. To compute the average turnover rate of an enzyme, only the times of the trajectory during which the enzyme is active are taken into account.

The fluctuations in single molecule turnover rates are stochastic in nature and indicate the enzyme switches between several active states and an inactive state. An analysis of the on-times (duration in which enzyme is active) and off-times (duration in which the enzyme is inactive) was performed (SI 4). The histograms for D-galactal can be fitted to an exponential function to obtain an association rate constant of 10^2 $M^{-1}s^{-1}$. The dissociation rates calculated from the exponential fits are $7x10^{-3}$ s^{-1} for D-galactal and $1x10^{-2}$ s^{-1} for NpBHC and compare well with the pre-steady state experiments.

Inhibitor induced changes in activity can be easily identified in the steady state experiments. In a steady state experiment, for a sequential binding and release model the enzyme switches between any two adjacent states depicted in scheme 1 upon an inhibitor binding or release event. On the other hand for a cooperative binding and release model, the enzyme switches between the first and last states in scheme 1. To identify any relationship between the activity of an enzyme before and after inhibitor binding, scatter plots of the pre and post activities are plotted. The first binding or unbinding event was considered for each trajectory in the steady state experiment. To identify the event of inhibitor binding or release, we looked for the first time point at which the enzyme switches between two activity levels. T(i) is the average turnover of the enzyme before inhibitor binding and T(i+1) is the average turnover of the enzyme after inhibitor binding and before a second binding event. A cross-correlation of T(i+1) with T(i) is shown in Figures 3a and b for D-galactal and NpBHC, respectively. In the case of D-galactal, the turnovers are only weakly correlated (spearman correlation coefficient = 0.20, $p<0.05$). Therefore the new activity state induced by D-galactal is relatively random. However, in the case of NpBHC, a significant positive correlation can be seen (spearman correlation coefficient = 0.674, $p<0.05$). Therefore, an enzyme with low activity before NpBHC binding is more likely to have low activity after NpBHC release and vice versa. In other words, the enzyme seems to have a memory of its activity before inhibitor binding. This result supports the hypothesis that D-galactal binds to β-galactosidase in a cooperative manner and alters its conformation significantly compared to NpBHC, which binds in a sequential manner.

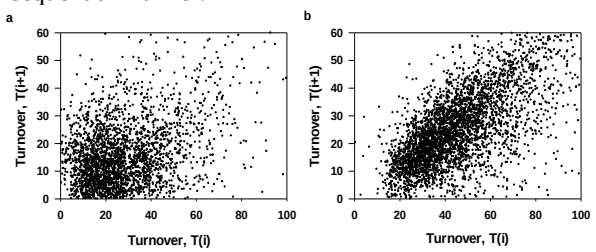


Figure 3. (a) and (b) Cross-correlation plots of substrate turnover rates of the adjacent activity levels of individual β-galactosidase molecules. T(i) is the activity before inhibitor induced change in activity and T(i+1) is the activity after the inhibitor induced change. The scatter plot for D-galactal shows that there is no relation between the activities (a), while that for NpBHC shows a positive correlation (b).

Static heterogeneity is a well established feature in the activity of β-galactosidase[5]. Considering that static heterogeneity originates either from translational errors or different conformational states of the enzyme, it is reasonable to anticipate that the extent of interaction of individual enzymes with the inhibitor will also have a broad distribution. To quantify the inhibitor interaction with the enzyme an autocorrelation analysis can be performed as described in

Reference 21 to extract the exchange rates of the inhibitor with the enzyme (SI 5). Exchange rate of the inhibitor is the sum of the on and off rates and is therefore a better indicator of the rate of inhibitor binding and release per unit time rather than counting the number of binding and release events for each trajectory.

To study the static heterogeneity of inhibitor interaction with the enzyme an autocorrelation analysis is performed on the steady state turnover rate trajectories (SI 5). The autocorrelation curves for both D-galactal and NpBHC fit well to a mono exponential decay (Figure S5.1a). For a single step binding and release, the autocorrelation is expected to have a mono-exponential decay. However, for a multi-step binding and release scheme, the autocorrelation of the turnover rates is expected to be a multi-exponential decay and should fit to a stretched exponential decay equation $C_m(t) = C_m(0)\exp[-(k_c \times t)^{\beta}]$. From the sequential release kinetics evident in pre-steady state experiments, the autocorrelation curves for activity traces of steady state NpBHC experiments were expected to fit to a stretched exponential decay equation. When plotted, the majority of them could be fit better to a mono exponential decay curve (β=1). It is possible that we do not have the necessary dynamic range of lag times to be able to observe a stretched exponential decay profile. To obtain the average exchange rate, the autocorrelation curves of thousands of enzymes (4999 for D-galactal and 2993 for NpBHC) were averaged and fit to a mono-exponential decay (Figure S5.1b). The exchange rates were computed to be 6.6×10^{-3} s^{-1} for D-galactal and 4.7×10^{-3} s^{-1} for NpBHC. The inhibitor exchange rates of individual β-galactosidase molecules show a broad distribution (Figure S5.1c) as expected.

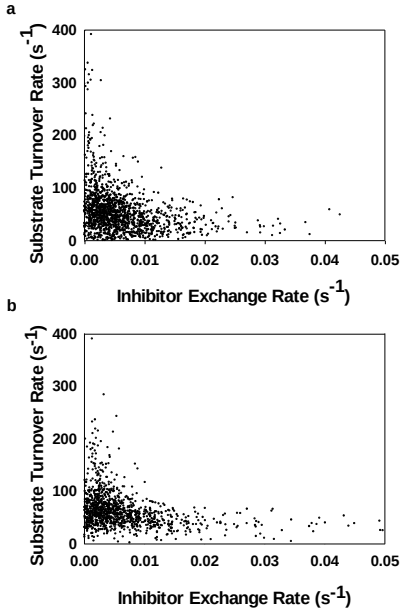


Figure 4. Cross correlation plot between the average substrate turnover rate and the inhibitor exchange rate computed for each β-galactosidase molecule under steady state conditions with two tight binding inhibitors (a) D-galactal (n = 1476) and (b) N-p-bromobenzylamino-hydroxymethyl-cyclopentanetriol (n = 1109).

Thus static heterogeneity can be observed for the inhibitor exchange rate as well as the substrate turnover rate. By measuring both parameters in the same experiment, the relative variation of these parameters across an enzyme population can be measured. The average substrate turnover rate computed for each enzyme molecule in the presence of the inhibitors was plotted against the inhibitor exchange rate calculated for the same molecule in a steady state experiment (Figure 4a and b). A negative correlation can be observed for both inhibitors – D-galactal ($p<10^{-25}$, Pearson's product moment correlation = -0.34) and NpBHC (negative correlation, $p<10^{-24}$, pearson's product moment correlation = -0.31).Therefore, enzyme molecules that have a high substrate turnover rate have a low inhibitor exchange rate and enzyme molecules that have high exchange rate have low turnover rate.

NpBHC is thought to mimic the structure of transition state intermediate in the hydrolysis of galactopyranosides by β-galactosidase. For the transition state mimic, the inverse correlation between turnover rate and exchange rate can be explained as follows: Enzymes that have high inhibitor exchange rates stabilize the transition state excessively resulting in a lower turnover rate with the substrate. Conversely enzymes that have higher turnover rates stabilize the transition state to a lesser extent and therefore have a lower inhibitor exchange rate. D-galactal is a non classical inhibitor. It forms a glycosyl enzyme intermediate which is slowly hydrolysed to release 2-deoxygalactose. Thus the inhibitor exchange rate of D-galactal is a measure of the rate of hydration of D-galactal. In this case the cross correlation plot in figure 5a compares two different activities of the enzyme – hydration of D-galactal versus the hydrolysis of the substrate resorufin β-D-galactopyranoside. The enzyme employs a different mechanism for D-galactal hydration[24] when compared to the substrate hydrolysis. As a result, the individual enzyme molecules that are more efficient at catalyzing the substrate hydrolysis are less efficient at catalyzing D-galactal hydration and vice versa.

Therefore, for both NpBHC and Dgalactal, the enzyme active site is selective towards the inhibitor or the substrate. In the case of D-galactal, selectivity results from the heterogeneity in catalytic efficiencies of the enzyme molecules. In the case of NpBHC the selectivity arises from the heterogeneity in the transition state stabilization. The heterogeneities in the enzyme active site may be due to conformational differences or due to differences in the amino acids of the active site arising from transcriptional or translational errors or a combination of these factors.

Multiple substrates

To ensure that the results from the competitive inhibition studies were not experimental artefacts, we performed a similar experiment and studied the competition for the active site between substrate and a non-effector species. To this end, we compared the relative variation of activities of the single β-galactosidase molecules in the presence of two fluorogenic substrates.

The substrates also compete for the active site but are not expected to affect each other's turnover rate[30]. Single molecules of β-galactosidase were trapped in optical fibre wells in the presence of an excess of two different substrates—FDG and RDG— (Fig. 5a) and the competition between the substrates for the four active sites of β-galactosidase was studied by monitoring their relative turnover rates. Controls for determining signal leakage between the filter cubes were performed by omitting one of the substrates but imaging in both the cubes. The signal leakage between the filter cubes was low. Figure 5b shows a cross correlation plot between the net fluorescence generated from the two substrates, demonstrating a perfectly positive correlation between the two substrates. The

positive cross correlation shown in Figure 5b indicates that the enzyme activity state affects the turnover rates of both substrates similarly. Therefore, a molecule that has high activity for one substrate also has a high activity for the other substrate, providing strong evidence that the results we obtained with substrate and inhibitors are valid. Additionally this plot demonstrates that in the competition between the substrates the active site is not selective for either substrate. On the contrary, the competition between substrate and inhibitor is dictated by the active site, i.e. if the active site is more selective for the inhibitor then it is less selective for the substrate and vice versa.

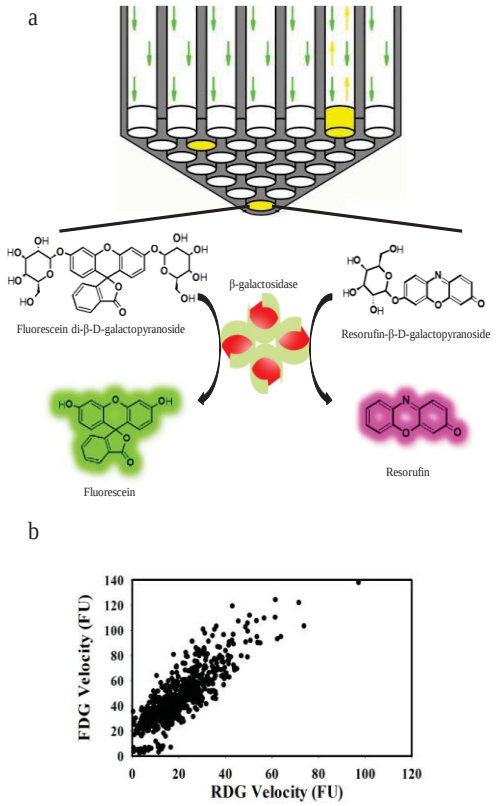


Figure 5. (a) Scheme for studying the relative kinetics of single β-galactosidase molecules in the presence of the two substrates FDG and RDG. Each well contains either 1 or 0 enzyme molecules and 10 μM of both FDG and RDG. The product generation was monitored by recording an image by alternating between the fluorescein and resorufin channels. The velocity for each reaction was computed by measuring the average change in fluorescence intensity per second recorded in the respective channel. (b) A cross correlation plot between the reaction velocities to compare the relative activities of individual enzyme molecules with respect to each substrate.

Conclusions

A single molecule technique was used to trap a large population of individual molecules of E. coli β-galactosidase and to study their activities in the presence of tight binding inhibitors. We studied and compared the stochastic binding and release of two different inhibitors of β-galactosidase —D-galactal and NpBHC. The inhibitor release kinetics of the two inhibitors was shown to be different. We were able to compare the effect of inhibitor binding on the enzyme conformation for the two inhibitors, which is impossible to differentiate in a bulk experiment. We demonstrated that D-galactal, a less potent inhibitor has a more drastic effect on the enzyme conformation than NpBHC, a more potent inhibitor. Furthermore, by using autocorrelation analysis on the observed turnover rates, the interaction of individual enzymes with the inhibitors and the substrate could be quantified. We demonstrated that the substrate turnover rate and inhibitor exchange rate are inversely correlated for both D-galactal and NpBHC.

While inhibitor binding and substrate turnover are mutually exclusive processes in competitive inhibition, we show that they are not independent of one another. The exchange rate of the inhibitor and the turnover rate of the substrate, for example, may be linked to each other via the enzyme conformation. The results of both competitive inhibition and a multiple substrate reaction also demonstrate the difference between substrate-inhibitor competition in which the active site is more selective for the substrate or the inhibitor and substrate-substrate competition in which the active site is not selective for either substrate.

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