5 mg of the enzyme purchased from Sigma-Aldrich was dissolved in 100 µL of 1xPBS and 1 mM MgCl₂ buffer and purified using HPLC. The A280 spectrum of the enzyme from the manufacturer obtained from a HPLC analysis using Zorbax GF450 column (1.0 ml/min flow and 45 bar pressure) is shown below in Fig S1.1. The peak at retention time 8.3 min was collected and concentrated using centrifugal filters (Amicon Ultra-0.5, 100Kda MWCO). The concentrated sample was run again in HPLC to verify the purity (Fig S1.1). Additionally the samples were characterized using a native gel (Novex 4-12% Tris-Glycine gels) (Fig S1.2). For further experiments, the concentration of the purified sample was determined using a Nanodrop (A280 measurements, extinction coefficient of β-galactosidase: 1142880 M⁻¹ cm⁻¹). The concentrations were also estimated using a Bradford assay and agree well with the Nanodrop measurements.

Figure S1.1—HPLC spectra of β-galactosidase purchased from Sigma-Aldrich. On the top is the spectrum of the commercially purchased β-galactosidase diluted in 1xPBS + 1mM MgCl₂ buffer. The HPLC spectrum shows the presence of a small percentage of impurities (1.5%) in the commercial sample. The fraction associated with the main peak at retention time of 8.5 min was collected and concentrated via centrifugal filters. This purified fraction was analyzed again on HPLC (bottom). The spectrum of the purified fraction showed negligible impurities (0.2%).
Figure S1.2—The HPLC purified β-galactosidase was analyzed using native gel electrophoresis (Novex® Tris-Glycine gels from Life Technologies). Lanes 1 and 2 of the gel shown above were loaded with 0.1x and 1x dilution of commercial β-galactosidase purchased from Sigma (5 mg dissolved in 200 ul of buffer). Lane 3 was loaded with NativeMark Protein standard. Lanes 4 and 5 were loaded with 0.1x and 1x dilution of the HPLC purified fraction of β-galactosidase.

SI 2: Bulk Studies to characterize inhibitors and obtain rate constants

Bulk experiments to determine the inhibition constant ($K_i$) and on and off-rates ($k_{on}$ and $k_{off}$) were performed on a Tecan microtiter plate reader. First, progress curves were obtained at different inhibitor concentrations by adding the enzyme to a mixture of substrate and inhibitor and recording the fluorescence intensity over time. These progress curves were fit to equation S1.1 to obtain $K_{obs,1}$ (Figure S2.1), the pseudo first order rate constant

$$[P] = \frac{v_0 - v_s}{k_{obs}} (1 - e^{-k_{obs}t}) + v_s t \quad \text{(Eqn. S2.1)}$$

where $v_0$ and $v_s$ are the initial and the steady state velocity respectively and [P] is the product concentration. The pseudo first order rate constant $K_{obs}$ is defined as:

$$k_{obs} = [I]k_{on} + k_{off} \quad \text{(Eqn. S2.2)}$$

where, $k_{on}$ and $k_{off}$ are the first order association rate constant and the dissociation rate constant, respectively. The $k_{obs}$ was computed at different inhibitor concentrations using (1) and plotted against the inhibitor concentration. This plot was fit to equation S2.2 to obtain $k_{on}$ and $k_{off}$ (Figure S2.2). The $k_{on}$ and $k_{off}$ for inhibition of β-galactosidase by NpBHC were determined to be $7 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ and $2.6 \times 10^{-3} \text{ s}^{-1}$, respectively.
Figure S2.1—Progress curve showing the slow binding inhibition of β-galactosidase by NpBHC. 360 pM of final concentration of the enzyme is added to a substrate-inhibitor mixture containing 100 µM of substrate and 20 nM of the inhibitor and the fluorescence intensity is recorded every 30 s for 30 min. The progress curve is fit to equation S1.1 to obtain the pseudo first order rate constant, $k_{\text{obs}}$. For 20 nM inhibitor, the $k_{\text{obs}}$ is 0.0162 s$^{-1}$.

Figure S2.2—The plot shows pseudo first order rate constant $k_{\text{obs}}$ obtained for different inhibitor concentrations. The plot can be fit to a straight line (eqn. S2.2) to determine $k_{\text{on}}$ and $k_{\text{off}}$.

**SI 3: Determination of “activity levels”**

In a pre-steady state experiment, an inhibitor release event is accompanied with a step increase in the turnover rate of the enzyme. The turnover rate between two inhibitor release events is stable and is defined as an “activity level”. The average turnover rate of each activity level is determined by taking the average turnover rate during the total time the enzyme dwells in that activity level. First, the time points at which an inhibitor release event occurs is determined by using a method based on student’s t-test. Then the turnover rates of all the time points between two subsequent inhibitor release events identified by the student’s t-test method are averaged to obtain the turnover rate of the activity level.

To employ the student’s t-test based method, the raw intensity data are first background subtracted and smoothed (Savitzky-Golay filter with a quadratic convolute and window length of 15) followed by conversion into turnover rates. To identify the inhibitor release events, the time points at which there is a sudden change in turnover rate have to be identified. At each time point the student’s t-test is used to compare seven values before and after the point. When the turnover rate is constant within the background standard deviation, the t-value is close to 0. When there is a sudden change in the turnover rate, the absolute t-value calculated at that point is high. When a profile of the t-values is plotted, a
spike in the t values indicates the inhibitor release events. By setting a threshold, the time points at which t-values are above the threshold can be assigned to inhibitor release events. Figure S3.1 demonstrates the use of the student’s t-test method to identify inhibitor release events.

Figure S3.1—Demonstration of the application of t-test method to identify inhibitor release events. (a) shows the smoothed background subtracted intensity profile of a pre-steady state experiment. The intensity profile is then converted into a turnover rate (b) by multiplying the derivative of (a) by a calibration constant. The t-test is then applied to the turnover rate profile in (b) and the t-values are plotted in (c). By setting a threshold (blue line in (c), we can identify the inhibitor release events. Multiple points can be seen below the threshold for each inhibitor release event. The local minimum is assigned to an inhibitor release.

The t-test was also used to identify an inhibitor binding or release event in steady state experiments. In this case the t-value profile shows downward spikes for inhibitor release and upward spikes for inhibitor binding. Positive and negative thresholds are used to determine the inhibitor binding and release events respectively (Figure S3.2).

The t-test method was validated by application to a simulated data set consisting of background noise extracted from the turnover rate profiles of experimentally collected data with added steps (jumps in turnover rate) of known location and size. The limit of step size (i.e. the jump in turnover rate) that can be estimated using the t-test is $17 \text{ s}^{-1}$ and the minimum dwell time for a step (i.e. an activity level) to be detected is 225 s.

Figure S3.2—Demonstration of the t-test method to identify inhibitor binding and release events in a steady state experiment. (a) The smoothed fluorescence intensity trajectory from a steady state experiment. The fluorescence intensities are converted into turnover rates as shown in (b). The t-test is applied at each point in (b) where seven points before and after the point are compared using student’s t-test. The t-values are plotted in (c). An upward spike indicates an inhibitor binding event, while a downward spike indicates an inhibitor release event. To accurately determine the events, the t-value profile is thresholded and the local maxima or minima beyond the thresholds are assigned to the binding or release events.
SI 4: Determination of on and off times

To determine the on and off times in the pre-steady and steady state experiments, a velocity thresholding method was used. The turnover rate profile obtained in the t-test method was further smoothened using a boxcar filter (window length 5). A predetermined threshold was used to determine if the enzyme was in an on state or off state. In the turnover rate profile, all the points that are above the threshold are considered on and all the points below the threshold are considered off.

For the steady state experiments, the dwell time in each state as determined by the thresholding method provides the on and off times. To determine the off times for the pre-steady state experiments, the thresholding method was used to identify the first time point after which the turnover rate is above the threshold. This time point gives the off-time for the profile. Using the velocity thresholding method the on-times and off-times in the steady state experiments of NpBHC and D-galactal were computed. Figure S4.1 shows the on-time and off-time histograms.

The histograms for D-galactal can be fitted to an exponential function to obtain an association rate constant of $10^3 \text{ M}^{-1}\text{s}^{-1}$ (Figure S4.1a). Histograms of the off-times (duration during which the enzyme is inactive) are plotted in Figure S4.1b and can be fit to an exponential decay as expected for a one step reaction. The dissociation rates calculated from the exponential fits are $7 \times 10^3 \text{s}^{-1}$ for D-galactal and $1 \times 10^2 \text{s}^{-1}$ for NpBHC. The on-time histogram for NpBHC shows a rise and decay profile as inhibitor binding is a multi-step process (Figure S4.1a). Assuming identical rates for each step of the multistep process, the on-time histogram is fit to the equation

\[
y = \frac{k^4 x^3}{4!} e^{-kx}
\]

Eqn. S4.1

Where $k$ is the on-rate and is calculated to be $4.1 \times 10^3 \text{s}^{-1}$. The association rate constant is calculated to be $8.2 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$.
SI 5: Autocorrelation Analysis

We determined the inhibitor exchange rates through the normalized autocorrelation function, $C_{(m)T}$, of the substrate turnover dwell times\textsuperscript{4,5} (Eq. 1):

$$C_{(m)T} = \frac{\langle \Delta T(0) \Delta T(m) \rangle}{\langle \Delta T^2 \rangle} = \frac{\sum_i \Delta T(i) \Delta T(i + m)}{\sum_i \Delta T^2(i)}$$

(Eqn. S5.1)

where $m$ is the time between two measurements, $T$ is the substrate turnover rate, $\Delta T(m) = T(m) - \langle T \rangle$, and the brackets denote averaging along a time trace. $C_{(m)T}$ has the following meaning for steady-state inhibition experiments: In the absence of inhibitor, $C_{(m)T}$ has the following meaning for steady-state inhibition experiments: In the absence of inhibitor, $C_{(m)T} = 1$ and $C_{(m)T} = 0$ with $m > 0$, as the substrate turnover rates are long-lived and do not fluctuate. In the presence of inhibitor, $C_{(m)T}$ follows a single-exponential decay, if we apply classical chemical kinetics. The initial amplitude ($m = 1$) reflects the variance of the substrate turnover rates, $\langle \Delta T^2 \rangle$, and the decay rate indicates how rapidly an individual enzyme molecule binds ($k_{on}$) and unbinds ($k_{off}$) inhibitor molecules. Thus, the data points of the substrate turnover autocorrelation function of each individual β-galactosidase molecule can be fitted to Eq. 2 to determine the inhibitor exchange rate $k_c$:\textsuperscript{1}

$$\langle \Delta T(0) \Delta T(m) \rangle = (\Delta T^2)e^{-k_cm}$$

(Eqn. S5.2)

$$k_c = k_{on}[I] + k_{off}$$

(Eqn. S5.3)
Figure S5.1—(a) Normalized autocorrelation of the substrate turnover rates shown in Figure 3b, computed as described in the data analysis. (b) Averaged autocorrelation function of the steady state turnover profiles of thousands of enzymes (4999 for D-galactald and 2993 for NpBHC). (c) Histogram of Inhibitor exchange rates for steady state reaction of β-galactosidase with D-galactal and NpBHC. The same data analysis was performed on a single molecule experiment of β-galactosidase in the absence of inhibitor and the extracted ‘inhibitor exchange rates’ are shown (No Inhibitor).

Figure 3b in the manuscript for example shows the noisy profile of substrate turnover rates of four individual enzyme molecules, where distinct states of activity can be observed. A plot of the corresponding autocorrelation function of the turnover rates to extract the inhibitor exchange rates is shown in Figure S5.1a. The autocorrelation function can be fit to a mono-exponential decay as shown. Histograms of the inhibitor exchange rates calculated from individual trajectories using Eqn. S5.2 are shown in Figure S5.1c. The exchange rate computed in the absence of inhibitor is also shown.

References