Ever-fluctuating single enzyme molecules
English, Brian P.; Min, Wei; van Oijen, Antonius; Lee, Kang Taek; Luo, Guobin; Sun, Hongye;
Cherayil, Binny J.; Kou, S.C.; Xie, X. Sunney
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Supplementary Methods

Enzyme functionalization and characterization

E. coli β-galactosidase (Grade VIII, Sigma) is purified using a Zorbax GF-250 size-exclusion HPLC column (Agilent) in pH 7 phosphate (dibasic) buffer containing 10 mM MgCl₂ and 145 mM NaCl. The enzyme is concentrated using a 10K centrifuge filter (Centricon) at 4°C to a concentration of 3µM and is then coupled to a 4 kDa biotin-PEG-maleimide linker (Nektar Pharmaceutical). The maleimide moiety of the linker can react specifically with a surface-accessible cysteine. Incubation with 4 µM of the bi-functional linker is conducted at room temperature for three hours. The reaction is quenched using 10 mM β-mercaptoethanol (Sigma). The aliquoted enzyme is stored in 50% glycerol buffer at −80 °C.

Large streptavidin-coated polystyrene beads (1 µm diameter, Bangs) allow for easy separation of biotin-linked enzyme from unlinked enzyme and were used to determine how many reactive PEG-biotin linkers are coupled to an enzyme molecule. After a short incubation of an aliquot of enzyme with an excess of beads, all biotin-functionalized β-galactosidase will be linked to the beads and hence biotin-functionalized enzyme can easily be separated from solution via centrifugation. Using a fluorometer, the ratio of biotinylated to non-biotinylated enzyme was determined by comparing the enzymatic activity on the beads to the activity of non-biotin-functionalized enzyme remaining in solution. Both activities were measured in buffer containing 200 µM RGP. The ratio of biotinylated to non-biotinylated enzyme was determined to be 1:3. Hence it is unlikely that one tetrameric enzyme has more than one biotin-linkers. We conclude that only one of the subunits is tethered to a bead.

We observed that the enzymatic rate of the enzyme before and after immobilization to the surface of the beads are the same, which proves that the enzyme is unperturbed after being linked to the surface with the long and flexible PEG linker (see Supplementary Fig. 3).

Flow chamber

A flow cell is prepared by carving out a 7 mm wide channel in 100 µM thick adhesive spacer (Grace Bio Labs) that is sandwiched between a functionalized coverslip and a quartz microscope slide. The quartz microscope slide has two holes that accommodate polyethylene tubing used for easy buffer exchange. The silanization reagent 3-aminopropyltriethoxysilane (Sigma) is used to amine-functionalize coverslips that have been thoroughly cleaned using alternating rounds of sonication in ethanol and 1M sodium hydroxide. After a 5 min incubation at pH = 8.2, the silanization reagent is cured onto the slip at 120 °C for 30 min. The amine-functionalized coverslips are then incubated with amine-reactive PEG and amine-reactive biotin-PEG at a ratio of 100:1 (both from Nektar) for 3 hours. The layer of PEG makes the coverslip more hydrophilic and hence prevents unspecific hydrophobic surface interactions. The biotinylated coverslips are stored under vacuum until use.

Intensity thresholding

To differentiate product fluorescence spikes from background noise, we first construct the intensity histogram of the time trace, as shown in Supplementary Figure 1. The histogram is composed of two contributions: a background peak and a signal peak, each fitted well with a Poisson distribution. The intersection between the two fitted
Poisson distributions sets the threshold $I^*$, above which a fluorescence spike is considered to arise from a enzymatic turnover.

**Determination of ensemble MM-coefficients**

We determine initial enzymatic velocity as a function of RGP concentration by recording the increase in fluorescence using a fluorometer for two biotin-linked β-galactosidase concentrations: 11 pM and 53 pM. Newly purified RGP is diluted to concentrations of 10, 25, 50, 100, 200 and 250 µM (at 53 pM β-galactosidase), or 25, 75, 150, 200, 250 µM RGP concentration (at 11 pM β-galactosidase). Two Lineweaver-Burke plots are shown Figure 2b in the main text. The slope of a Lineweaver-Burke plot is $K_M/v_{\max}$, the y-intercept is $1/v_{\max}$ and the extrapolated x-intercept is $-1/K_M$. Values of $K_M$ and $v_{\max}$ are derived from a global weighted least-squares fit of both curves and a $v_{\max}$ value of 740 ± 60 s$^{-1}$ and a $K_M$ value of 380 ± 40 µM is obtained.

**Diffusion in poly(ethylene-glycol)**

To assure efficient bleaching, all experiments are conducted in PEG (8000 MW, Sigma) to prolong the resident time of a resorufin molecule. Unlike glycerol, PEG acts as a macroviscogen, and increases the viscosity at large dimensions by forming pores within which the diffusion of small molecules is unaffected$^{1,2}$. Hence PEG does not affect the Michaelis $k_2$ and $K_M^2$. In ensemble experiments conducted on the fluorometer, we found increasing PEG up to 20 % (w/w) concentrations did not substantially affect enzymatic turnover rates. For all experiments conducted between 20 and 100 µM RGP, the final PEG concentration is adjusted to 10 % (w/w). For all experiments conducted at 380 µM, the PEG concentration is increased to 15 % (w/w).

**Control experiments**

An important control is to be able to turn off enzymatic activity of a single enzyme molecule. To do so, we used a competitive inhibitor phenylethyl-β-D-thiogalactopyranoside (PETG, Molecular Probes) and observed that enzymatic turnovers were halted (see Fig. 1c in main text). This proves that signal is solely due to enzymatic turnovers.

It is also important that the enzyme has to bind to the bead specifically through the biotinylated linker, rather than through nonspecific binding. In another control experiment, beads are incubated with non-biotinylated β-galactosidase at high concentrations, and we observe no beads exhibiting fluorescence signals (hence enzymatic activity).

**Intensity autocorrelation analysis**

Supplementary Figure 6 illustrates the autocorrelation functions of three hypothetical kinetic scenarios: an enzyme with only one constant turnover rate produces a flat intensity autocorrelation curve (Supp. Fig. 6A). If a single exponential decay is observed in the intensity autocorrelation curve, the enzyme must have two interconverting conformers with differing turnover rates. Its decay rate then reveals the sum of the two interconversion rates (Supp. Fig. 6B). More and more interconverting conformations will result in an autocorrelation function that is increasingly more multi-exponential (Fig. S6C).
Autocorrelation analysis has the advantage that it is insensitive to background noise and spikes due to fluorescent molecules diffusing through the probe volume. This is illustrated by a simulation in which both counting noise and background spikes are artificially added into the intensity time trace (Supp. Fig. 6B, right panel). For simplicity, the simulated enzymatic time trace has only two conformers. As expected, in the absence of noise, $C(t)$ is a single-exponential with a decay time-constant equal to the inverse sum of the two interconverting rate constants. With the addition of both background noise and diffusing molecules, there is a spike at $t=0$ with an amplitude given by the variances of all sources of intensity fluctuations. For $t>0$, the functional form of the autocorrelation curve is unaffected by the counting and background noise.

**Monte-Carlo simulations of multiple conformers**

The experimentally observed $C(t)$ at high substrate concentration can be phenomenologically fit well to a stretched-exponential function ($\beta = 0.4$) (see black trace in Fig. 5a in main text).

With five interchanging conformers, the autocorrelation curves $C(t)$ from time-traces obtained by Monte-Carlo simulations can never be fit well to a stretched-exponential function (Supp. Fig. 6C top panel). Only when the interconversion (0.13 s$^{-1}$ to 10 s$^{-1}$) and turnover rates (10 s$^{-1}$ to 1000 s$^{-1}$) are chosen to be broadly distributed can $C(t)$ be fit to a sum of four exponential decays corresponding to the four eigen-values of the rate-matrix ($e_1$ to $e_4$). The switching and turnover rates are tabulated.

With ten interchanging conformers, a reasonable fit to a stretched exponential $C(t)$ ($\beta = 0.5$) to the autocorrelation curve of the simulated time-trace can only be obtained with a small pre-selected set of interconverting (0.11 s$^{-1}$ to 10 s$^{-1}$) and turnover rates (20 s$^{-1}$ to 10000 s$^{-1}$) (Supp. Fig. 6C bottom panel). The switching and turnover rates are tabulated. However, the simulated time traces look distinctly different from the observed ones (Fig 1d, main text) as the simulated turnover time-traces under this condition are composed of prolonged segments of very high (10000 s$^{-1}$) and very low (30 s$^{-1}$) enzymatic activity.

These Monte-Carlo simulations demonstrate that a large number (rather than a few) of interconverting conformers are necessary to explain the highly stretched autocorrelation functions of turnover velocity observed experimentally.

**Autocorrelation function of $k$**

In addition to $C(t)$ discussed in the main text, we can obtain autocorrelation function $<\delta k(0)\delta k(t)>$ according to:

$$<\delta k(0)\delta k(t)> / \langle k \rangle^2 = N(t) / \langle k \rangle - 1$$

where $N(t)$ is the probability density function of any two turnover events separated by time $t$ regardless of the number of turnovers in between. In the absence of $k$ fluctuation, $N(t)$ is a constant $k$, and $<\delta k(0)\delta k(t)>$ is zero for all times.

Supplementary Figure 7 depicts $<\delta k(0)\delta k(t)> / \langle k \rangle^2$ for the time trace at 100 µM RGP concentration, which exhibits the same temporal decays of the corresponding $C(t)$ and $C_\tau(t)$, but without sacrificing temporal resolution as for $C_\tau(t)$. The consistency at 100 µM RGP indicates that at an even higher concentration, such as 380 µM, $C_\tau(t)$ must also reflect fluctuations in $k(t)$. At 380 µM RGP, $<\delta k(0)\delta k(t)>$ cannot be evaluated directly because individual turnovers cannot be resolved.
References


