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Manipulating and Probing Enzymatic Conformational Fluctuations and Enzyme-Substrate Interactions by Single-Molecule FRET-Magnetic Tweezers Microscopy

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Enzyme-substrate interaction plays a critical role in enzymatic reactions, forming the active enzyme-substrate complex, the transition state ready to react. Studying the enzyme-substrate interaction will help the ultimate molecular-level characterization of the enzymatic transition state that defines the reaction pathway, energetics, and the dynamics. In our initial effort to experimentally investigate the enzyme-substrate interactions and the related conformational fluctuations, we have developed a new approach to manipulate the enzymatic conformation and enzyme-substrate interaction at single-molecule level by using a combined magnetic tweezers and simultaneous fluorescence resonance energy transfer (FRET) spectroscopic microscopy. By a repetitive pulling-releasing manipulation of a Cy3-Cy5 dye labeled 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase (HPPK) molecules under the conditions with and without enzymatic substrates, we have probed and analyzed the enzymatic conformational dynamics. Our results indicate that the enzyme conformational flexibility can be regulated by enzyme-substrate interactions: (1) enzyme at its conformation-perturbed state has less flexibility when binding substrates, and (2) substrate binding to enzyme significantly changes the enzyme conformational flexibility, an experimental evidence of so called entropy trapping in enzyme-substrate reactive transition state. Furthermore, our results provide a significant experimental analysis of folding-binding interactions of the enzyme-substrate interactions, a dynamic nature of the enzymatic active transition state formation process.

Conformational change of protein molecules is often critical for the biological functions, affecting the affinity and selectivity of protein-protein and protein-ligand interactions, and further regulating the catalytic activity of enzymatic reactions.[1-4] For example, an enzyme can have different activities with different conformations.[5-7] Thus, manipulating protein conformations can be an effective approach to study the relationship between protein conformation and function.[8-30]

One of the central questions in protein functions is the impact of ligand binding to conformational fluctuation or conformational flexibility changes of protein molecules, especially enzyme-substrate interaction.[3,64-65,70] The answer of this question serves a critical understanding of the enzyme-substrate interactions and the enzymatic active transition state formation. In recent years, a number of novel single-molecule approaches combining single-molecule optical spectroscopy with mechanical force manipulation approaches have been developed to achieve protein conformational manipulation, such as atomic force microscope (AFM), optical tweezers, and magnetic tweezers, etc.[9-11,21-36] Here we report our newly developed approach using magnetic tweezers correlated with single-molecule FRET spectroscopy to study ligand-binding impact on enzymatic conformation by force manipulating single enzyme molecule conformation with simultaneous optical observation of the enzyme conformational fluctuations under different conditions of with and without enzymatic substrate.

Compared with other approaches for manipulating single protein molecules, such as AFM or optical tweezers, magnetic tweezers has a number of desirable and complimentary specificities: (1) magnetic tweezers can apply a pulling force either in a fine scale as small as sub-picoNewton[37] or in a relative large scale close to nanoNewtons;[38] (2) magnetic tweezers does not require physical contact or chemical contact to target molecules; (3) magnetic tweezers does not induce either photo-damage to the sample or a photon background noise to a correlated simultaneous single-molecule

spectroscopic measurement; (4) magnetic tweezers allows manipulating conformation of a large number of molecules simultaneously as long as the molecules are tethered to paramagnetic micro beads. These specificities make the magnetic tweezers approach promising for protein conformational manipulation. Since 1990s, extensive studies on manipulating single biological molecules by using magnetic tweezers have been reported.[39-45,56] The applications of magnetic tweezers manipulating biological molecules have been extended from DNA wringing[46,47] to polymer protein molecules pulling[48, 49]. The correlated theoretical simulations have also been developed in recent years.[48,49] Nevertheless, to our knowledge, conformational manipulation by magnetic tweezers and correlated simultaneous single-molecule FRET spectroscopic analysis of a single protein molecule has not been reported.

HPPK is an 18 kDa 158-residue monomeric enzyme protein molecule with the biological function to catalyze the transferring of pyrophosphate from ATP to 6-hydroxymethyl-7,8-dihydropterin (HP), releasing adenosine monophosphate (AMP) and 6-hydroxymethyl-7,8-dihydropterin pyrophosphate (HPPP) as products.[50-53] We choose Cy3-Cy5 dye labeled HPPK as a model system to study the effect of external force triggering on enzymatic conformational dynamics by using combined magnetic tweezers manipulations and correlated FRET measurement in the solution with and without enzymatic substrates.

As shown in Figure 1, the fluorescent dyes, Cy3 and Cy5 as FRET donor and acceptor, were labeled to the mutated amino acid residue 48 on loop 2 and residue 151 close to the active site of the enzyme, respectively. The Cy3/Cy5 fluorescent dye pair was labeled to the mutated enzyme at residue 48/151 non-specifically, and the labeling has no significant impact on the enzymatic activities.[54] The HPPK molecules were bound to the glass cover slip at one end by 3-aminopropyltriethoxy-silane (TESPA)-Dimethyl Suberimidate•2HCl (DMS) linkers and linked to a super-paramagnetic bead (Dynabeads® MyOne™ Streptavidin T1, 1.05-µm diameter, Invitrogen Company) at the other end via biotin-streptavidin bond. Protein immobilization was carried out through a routine procedure (for details, see Supporting Information, Figure S2). Briefly, a clean glass coverslip was immersed overnight in NaOH-ethanol solution. The coverslip was next washed by distilled water, blow-dried by air flow, and incubated with a DMSO solution containing a mixture in 10% concentration consisting of TESPA and isobutyltrimethoxysilane in 1:10000 ratio overnight. The coverslip was then washed by distilled water and consecutively transferred and incubated for 4 hours in each system below: 15 mL PBS buffer solution PH=8.0, containing 10nM Dimethyl Suberimidate•2HCl (DMS•2HCl); 15mL PBS buffer solution PH=7.4, containing 1nM HPPK; 15 mL PBS buffer solution PH=7.4, containing 10 nM NHS-PEO12-biotin; 15 ml PBS solution PH=7.4, containing 1µl magnetic beads stock solution which is commercial available. The low concentration of each solution was to make sure that the distribution of protein molecules on cover glass is adequately separated so that one bead does not attach to multiple protein molecules. Meanwhile, low concentrations of TESPA are used to ensure that immobilized protein molecules are distributed separately enough from each other for obtaining single molecule FRET images (For details, see Supporting Information). We note that either biotin or DMS can only be tethered to a HPPK molecule via connection with lysine in the amino acid sequence, which leads to multiple possible tethered condition of the protein molecule to coverslip or magnetic beads (Details in Figure S1). However, in each FRET measurement, we focused on a specific individual HPPK molecule during our repetitive manipulation by magnetic tweezers. Consequently, although we did not necessarily pinpoint that a pair of specific lysine residues tethered to a specific protein molecule, our observation of the reproducibly FRET changes under periodically applied magnetic field demonstrates that successful single-molecule level protein conformational manipulation is achieved.

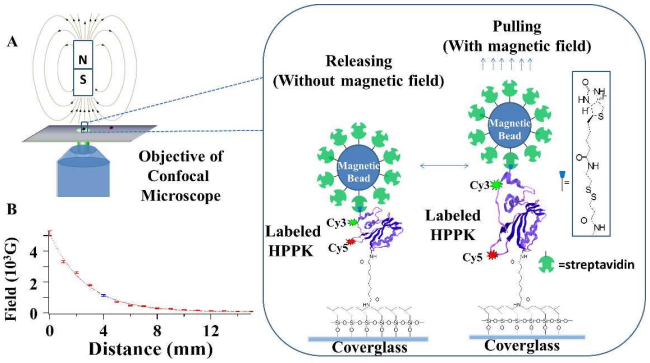

Figure 1. A conceptual scheme of our experimental system. **(A)** Cy3-Cy5 labeled HPPK kinase molecules are tethered on a modified glass coverslip that was positioned in a buffer solution chamber. The inset panel presents the concept of the conformational manipulation of a single HPPK molecule by magnetic tweezers. The cover glass is treated by 3-aminopropyltriethoxy-silane (TESPA) and isobutyltrimethoxysilane in 1:10000 ratio. Dimethyl Suberimidate•2HCl (DMS) is used as cross linker to immobilize HPPK protein molecule on the treated cover glass. The immobilized HPPK molecules are tethered through NHS-PEO_{12}-biotinlinking the lysine residue of HPPK to the streptavidin-coated magnetic beads. **(B)** Magnetic Field-Distance curve of the magnet used in experiment. The blue data point indicates the position of the magnet in our experiments: the magnet is set 4 mm above the sample plane to generate a magnetic field with approximately 1100 Gauss.

The FRET measurements were carried out by using an inverted confocal microscope (Axiovert 200, Zeiss) with a 532 nm CW Crystal laser for optical excitation, and the details for the single-molecule FRET imaging microscopic setup is described in our previous publications.[54, 55] We conducted the single-molecule FRET measurements with simultaneous magnetic tweezers pulling of HPPK enzyme molecules in PBS buffer solution under the conditions with and without the enzymatic reaction substrates. In our experiments, the immobilized enzyme molecules were immersed in a solution containing 50 mM pH=7.4 PBS buffer solution as imaging buffer and 1 mM 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic (Trolox) solution as oxygen scavenger to protect dye molecules from photobleach. The essential component of our magnetic tweezers device is a homemade cone-shape permanent magnet mounted on an independent 3D translational movement stage that controls the movement of the magnet (Figure 1).

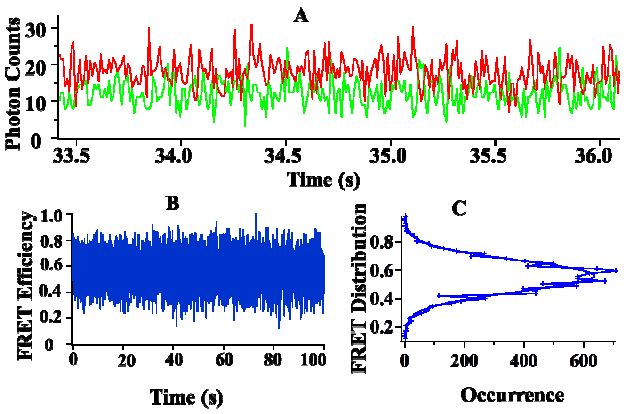

Figure 2. Single-molecule FRET data obtained from a Cy3-Cy5 labeled HPPK under magnetic field manipulation. **(A)** A portion of a pair of single-molecule fluorescence intensity time trajectories of FRET donor (green line) and acceptor (red line). **(B)** The FRET efficiency calculated from the pair of fluorescence intensity trajectories of the donor and acceptor in A. **(C)** The FRET efficiency distribution deduced from B.

Figure 2A shows a pair of FRET donor-acceptor (D-A) fluorescence intensity trajectories from a single Cy3-Cy5 labeled HPPK molecule under force manipulation by magnetic tweezers. The FRET efficiency E is calculated from equation 1, in which I_D and I_A stand for the emission intensity of donor and acceptor, respectively. Figure 2C, the histogram of the FRET efficiency, shows the distribution of FRET efficiency.

$$E=I_A/(I_D+I_A) \quad (1)$$

The FRET efficiency reflects the distance between the two dyes labeled on protein molecules, described by equation 2, in which R is the distance between donor Cy3 and acceptor Cy5 while R_0 is a constant determined by the transition donor-acceptor dipole–dipole interaction. In this experiment, when a pulling force is applied by the external magnetic field, we are able to probe the conformational changes from the simultaneous single-molecule FRET efficiency trajectories.

$$E_{FRET}=1/[1+(R/R_0)^6] \quad (2)$$

Mechanical force from external magnetic field is applied on a targeted protein through a paramagnetic bead linked covalently to the single protein molecule. To quantitatively understand the force that applied on protein molecules by the magnetic tweezers, we note that there are a number of specific approaches to estimate the mechanical forces applied though a magnetic field on a paramagnetic bead: (1) Measuring and model analyzing the Brownian motions of a tethered paramagnetic bead;[44] (2) Monitoring the dragging motion of a small number of magnetic beads in liquid environment with known viscosity;[37,40] (3) Observing the displacement of a micropipette with a magnetic bead attached at its end, etc.[37] Different methods for measuring torque on magnetic beads have also been developed.[57] Nevertheless, each of the estimation approaches bears specific merit of estimation with certain error bars. We have applied a model analysis based on the measured magnetic field strength curve (Figure 1B) as a function of the distance between the magnetic tip and the sample surface.

We calibrate the applied force by estimating the magnetic field gradient to get the magnetic moment of the beads tethered on the single protein molecule. For a magnetic bead in an externally-produced magnetic field $\boldsymbol{B}$, noting its magnetic moment as $\boldsymbol{m}$, then the potential energy $\boldsymbol{U}$ is:

$$\boldsymbol{U} = -\boldsymbol{m}\cdot\boldsymbol{B} \quad (3)$$

For a given magnetic bead, its magnetic moment $\boldsymbol{m}$ is the product of the volume magnetization $\boldsymbol{M}$ and volume V of the bead. Therefore, the force $\boldsymbol{F}$ that is applied on the magnetic bead can be calculated:

$$\boldsymbol{F} = -\nabla\boldsymbol{U} = -\nabla(-\boldsymbol{m}\cdot\boldsymbol{B}) = \boldsymbol{m}\cdot\nabla\boldsymbol{B} = \boldsymbol{M}V\cdot\nabla\boldsymbol{B} = \boldsymbol{M}V\frac{\partial\boldsymbol{B}}{\partial z} \quad (4)$$

In our experiments, the magnetic field applied is approximately 1100 Gauss. As an approximation, we only consider the magnetic field gradient in one direction perpendicular to the sample plane. Thus the field gradient can be estimated from the curve shown in Figure 1B. In this way the value of field gradient is calculated to be 55±15 T/m. When calculating the field gradient, position error that up to 1mm is taken into consideration as uncertainty of distance between the magnet and the sample plane. The volume V of paramagnetic bead is $0.6\times10^{-18}m^3$, and the volume magnetization $\boldsymbol{M}$ is 43×10^3 A/m.[58] In our calculation, we have considered the factor that $\boldsymbol{M}$ here is the saturation magnetization, an approximation that may bring error less than 25%. Hence the force is calculated 1.4±0.4 pN from equation 4. The typical force applied to the targeted single-molecule HPPK proteins is roughly 1-3 pico-Newton that is weaker than a typical hydrogen bonding force of 6-9 pico-Newton.

Figure 3A shows the FRET efficiency distribution measured from a single HPPK enzyme under the enzymatic reaction conditions with the substrate of ATP and HP added in PBS buffer. With the magnetic field applied, the mean of the FRET efficiency is significantly shifted from 0.5 to 0.3, which suggests that the single-molecule HPPK enzyme is stretched out in conformation under the external pulling force. The result shown in Figure 3 demonstrates that our combined technical approach of magnetic tweezers correlated single-molecule FRET spectroscopy is sensitive and capable of manipulating and measuring molecule conformational changes simultaneously. To further demonstrate the reproducibility and effectiveness of the force manipulation of enzyme conformations by the magnetic tweezers correlated single-molecule FRET spectroscopy, we have conducted a repetitive force pulling and releasing manipulation of single HPPK enzyme molecules. Figure 3B shows that the single-molecule FRET efficiency toggles between 0.5 and 0.3 reflecting the enzyme conformational changes due to the manipulation by the external force pulling and releasing, demonstrating high reproducibility and feasibility of the force manipulation of the conformational changes of the single-molecule enzymes. It is intriguing that the FRET distribution shows a bimodal distribution pattern around efficiency value 0.2 when the enzyme molecule is pulled by magnetic force, which is most likely due to the force perturbation of the molecule, and the molecule still has significant conformational flexibility under the weak force manipulation. Nevertheless, the focus of this control experiment is to demonstrate the feasibility of the repetitive force manipulation of the overall enzyme conformational changes and distributions while the conformation fluctuations of the enzyme are still allowed and measurable.

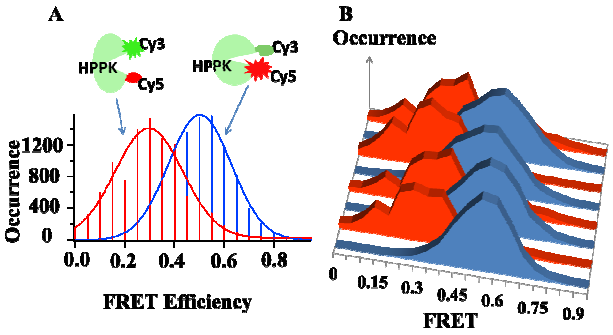

Figure 3. Repetitive force pulling and releasing manipulation of individual kinase enzyme molecules. **(A)** The FRET efficiency distributions of single HPPK molecules under a force pulling (Red) and releasing (Blue) manipulation. **(B)** The FRET efficiency response of a single HPPK protein molecule being repetitively toggled with (Red) and without (Blue) the external magnetic force. These FRET distributions are obtained from a series of continuous single-molecule FRET measurements with the substrate of ATP and HP added in the PBS buffer solution.

In an enzymatic reaction, the enzyme-substrate interaction is the crucial step determining the overall reaction dynamics as well as the reactivity and selectivity, according to the Michaelis-Menton mechanism and recent experimental and theoretical studies.[59-66] The enzyme-substrate complex formation can regulate both static and dynamic conformations of the enzyme, and the enzyme-substrate complex requires a specific molecular conformation to form an active enzyme-substrate complex state ready to react and convert the substrate to the product.

By probing the conformational fluctuations of single-molecule enzyme under the conditions of with substrate and without substrate, we have observed a significant change in conformational fluctuation distribution induced by the external force. Figure 4A and 4D show the enzymatic conformational distributions of HPPK in the buffer solution without the substrate, under the conditions of without (Figure 4A) and with (Figure 4D) the external pulling force, respectively. Figure 4B and 4E show the enzymatic conformational distributions in the buffer solution with the substrate of 100 μM ATP, 100 μM HP and under the conditions of without (Figure 4B) and with (Figure 4E) external pulling force manipulation, respectively. Comparing the distributions in Figure 4A and Figure 4B, measured under no external force manipulation, it is remarkable that the enzyme-substrate interaction narrows the conformational fluctuation range significantly, indicated by the smaller standard deviation of the FRET efficiency distribution (Figure 4B), which suggests that the enzyme-substrate interaction decreases the enzymatic conformational flexibility and the overall spatial accessibility. It is known that the enzyme-substrate interaction can narrow and limit the enzyme conformational flexibility and accessible space, according the well demonstrated conformational selection mechanism or induced fit mechanism.[67-73] However, due to the external force manipulations, the enzyme-substrate interaction is not able to cause a significant change in standard deviation of conformational fluctuation distributions, as shown in Figure 4D and Figure 4E, suggesting the external force manipulation provides a dominating impact on the enzyme conformational flexibility and limits the impact of the enzyme-substrate interaction on the enzyme conformational flexibility.

For more quantitative understanding of the impact of enzyme-substrate interaction on enzymatic conformation fluctuation and the impact of external force manipulation on the enzyme-substrate interaction, we further use the standard deviation of FRET efficiency distribution to quantitatively characterize the broadness of the FRET efficiency distributions as well as the conformational flexibility.[74,75] More flexible enzymatic conformation gives a wider enzymatic conformational fluctuation distribution in range, and a larger standard deviation of the conformational distribution. The results (Figure 4C and 4F) suggest that (1) the enzyme molecules without substrate have more flexible conformational fluctuations, which is indicated by the larger standard deviation in the FRET efficiency distribution; (2) the enzyme molecules with substrate interaction have more spatially confined conformational changes and less flexible conformational fluctuations, which is indicated by the smaller standard deviation in the FRET efficiency distribution; and (3) an external force pulling on an enzyme molecule decreases the impact of selective binding-folding enzyme-substrate interaction at the enzymatic active site. This attribution is further supported by the results measured under the conditions with and without substrate presence: the enzymatic conformational distributions under a pulling force perturbation (Figure 4D, 4E, and 4F) show less difference in the standard deviation in FRET efficiency comparing to the same standard deviation measured in HPPK without the pulling force perturbation (Figure 4A, 4B, and 4C) .

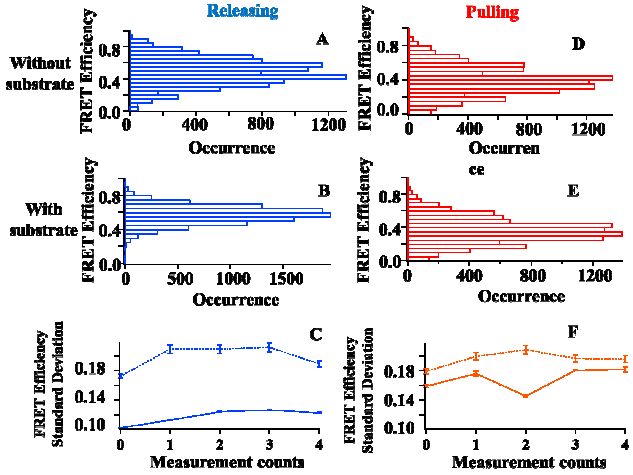

Figure 4. Perturbing and characterizing enzyme-substrate binding interaction by single-molecule FRET magnetic tweezers microscopy. **(A)** Distribution of FRET efficiency of a single apo-HPPK molecule. **(B)** Distribution of FRET efficiency of HPPK in ATP and HP substrate solution. **(C)** The standard deviation of FRET efficiency of single HPPK molecules measured under the conditions of with (solid line) and without (dashed line) substrate in buffer solution and without the force perturbation. **(D)** Distribution of FRET efficiency of a single HPPK molecule under magnetic force pulling and without substrate ATP and HP added. **(E)**

Distribution of FRET efficiency of a single HPPK molecule under magnetic force pulling in the solution with substrate ATP and HP added. (**F**) The standard deviation of FRET efficiency of single HPPK molecules measured under the conditions of with (solid line) and without (dashed line) substrate in buffer solution and with the force perturbation. The error bar in both figure 4C and 4F are forth central moment of FRET distribution. Evidently, under the force perturbation, the enzyme conformation is less sensitive to enzyme-substrate interactions comparing to the results in 4C when the measurement is under no external force perturbation.

Our results demonstrate that the enzymatic conformational fluctuation accessible space is strongly influenced by the enzyme-substrate interactions, which provides experimental evidence showing the critical role of the protein-ligand interactions in a possible conformation selection mechanism of enzyme-substrate complex formation. Conformations of protein molecules undergo dynamical fluctuations under physiological conditions, while the existence of ligands induces conformational regulation energetically and spontaneously favor to those conformations involving in ligand-active sites binding interactions.[76-79] Consequentially, the conformational distribution narrows down to a ligand-binding accessible conformational subset out of the broad conformational distribution of the apo HPPK enzymes without involving in protein-ligand interactions, as shown in Figure 4C and 4F.

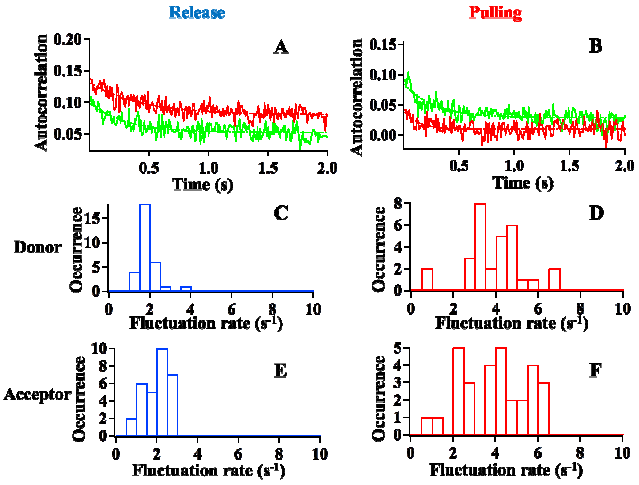

Figure 5. Conformational fluctuation rate distributions calculated from autocorrelation analysis of HPPK with substrate ATP and HP added. **(A)** Autocorrelation functions from FRET intensity trajectory measured under the condition of without magnetic pulling force. Green line indicates donor while red for acceptor. **(B)** Autocorrelation functions from FRET intensity trajectory measured under the condition of with magnetic pulling force applied. Green line indicates donor while red for acceptor. **(C and D)** Fluctuation rate distributions of FRET donor under conditions with and without magnetic pulling force. **(E and F)** Fluctuation rate distributions of FRET acceptor under conditions with and without magnetic pulling force.

To further characterize the changes in conformational dynamics with respect to the conformational flexibility and the accessibility of the conformations associated with the enzyme-substrate interactions, we analyze the autocorrelation functions of fluorescence fluctuation trajectories of our single molecule FRET measurements (Figure 5A and 5B), under the conditions of with and without the magnetic field, for both donor (Figure 5C and 5D) and acceptor (Figure 5E and 5F), while the HPPK molecule is with the substrate of ATP and HP added in the PBS buffer solution. Conformational fluctuation rate can be calculated from the exponential decay rate of autocorrelation function, and the essentially same decay rates between the autocorrelation functions of donor and acceptor in both A and B strongly indicate that the fluctuations are from the same origin, the single-molecular FRET. We have studied 30 different timing on FRET trajectories of one single molecule under both with and without magnetic pulling force conditions to have the distributions of conformational fluctuation rate as shown in Figure 5C, 5D, 5E and 5F. Figure 5C and 5D show the distributions of conformational fluctuation rate calculated from autocorrelation functions, and the distributions show a remarkable broadening, under the condition of with the magnetic pulling force, comparing to that of measured under without the magnetic pulling force. This result indicates an increase in distribution range of conformational fluctuation rate under the magnetic force pulling. Similar change triggered by magnetic tweezers pulling force also occurs consistently in acceptor fluctuation rate distributions (Figure 5E and 5F).

Such broadenings in the conformational fluctuation rate distribution are consistent with the results from the standard deviation analysis of FRET efficiency distributions. According to Figure 4C and 4F, it is the substrate binding interaction that leads to less flexible conformational fluctuations of HPPK protein molecule, while such interaction is weakened by applied external pulling force. Consequently, weakened enzyme-substrate interactions also result in an apparent broadening of conformational fluctuation rate distribution. Enzyme-substrate interaction is highly sensitive to the protein conformational perturbation by external pulling force. With the force pulling, such enzyme-substrate interaction is perturbed and weakened, releasing the protein from being constrained by ligand-binding interaction, resulting in a broader range of the enzyme conformational fluctuation rate.

Our results show that the small external force of about 1.4±0.4 pN can apparently impact the enzymatic conformational fluctuation distribution, and the enzymatic conformational flexibility or conformational fluctuation distribution is critical for enzyme-substrate interaction, thus the small external force can impact the interactions between enzyme and substrate. This low external pulling force is not sufficient to rupture the protein tertiary structures as the rupture force is at least 18 pN for HPPK[55] or even not sufficient to break hydrogen bonds as a typical hydrogen bonding force is about 4 pN and higher.[80] Therefore, the low external force we applied to an individual HPPK enzyme molecule likely only causes a deformation of tertiary structure of the HPPK enzyme molecule.

To illustrate the fact that the small external pulling force is capable of impacting the enzymatic function, such as enzyme-substrate interaction, we note that the external pulling force applied on an single enzyme molecule through the magnetic tweezers, even if the force is at similar scale competing with the thermal fluctuation forces, is an one-direction constant force that capable of deviating the conformational fluctuation energy landscape, leading to a deformation of the HPPK enzyme molecule. As an analogy, it is simply like a random walk on a titled energy landscape by an external and constant force field.

Evidently, the one-direction pulling force decreases enzymatic conformational flexibility and affecting the enzyme-substrate interaction impacting enzymatic function. Furthermore, we emphasize that this work only focuses on the understanding of the enzyme-substrate interactions in forming the enzyme-substrate reactive complex, which is the first step of an enzymatic reaction, and our future work will focus on identify and characterize the impact of the external force manipulation on the enzymatic reaction turnover activities.

Conclusions

In summary, we have demonstrated that our correlated single-molecule FRET-magnetic tweezers microscopy is capable of manipulating the conformation of single enzyme molecules, and in turn, manipulating the enzyme-substrate interactions, by applying and controlling a pulling force on single kinase molecules. Technically, the correlated magnetic tweezers single-molecule FRET spectroscopy is a potentially powerful tool to interrogate the protein conformational dynamics and the associated protein functions. Using our approach, we are able to interrogate the conformational selection mechanism by exam the conformation flexibility and conformational fluctuation accessible space when the enzyme is under interacting and not interacting with the substrate molecules. We have observed that the enzyme-substrate interaction provides a strong conformational selection effect through a folding-binding interacting process shifting the conformational fluctuation to more confined spatial range; whereas, under the force pulling, distorted enzyme conformation has a weaker interaction with the substrate, leading to a weak conformational selection effect and folding-binding interacting dynamics.

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Notes and references

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† Electronic Supplementary Information (ESI) available: molecular detail of HPPK sample system, sample preparation and instrumentation. See DOI: 10.1039/b000000x/

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