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Exothermicity Is Not a Necessary Condition for Enhanced Diffusion of Enzymes

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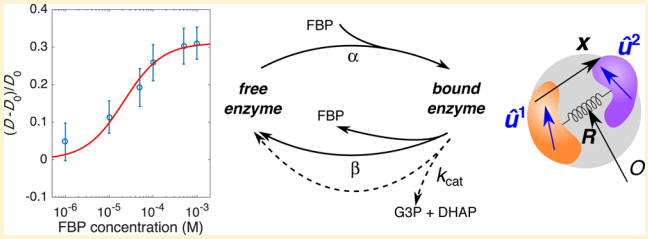
Supporting Information

ABSTRACT: Recent experiments have revealed that the diffusivity of exothermic and fast enzymes is enhanced when they are catalytically active, and different physical mechanisms have been explored and quantified to account for this observation. We perform measurements on the endothermic and relatively slow enzyme aldolase, which also shows substrate-induced enhanced diffusion. We propose a new physical paradigm, which reveals that the diffusion coefficient of a model enzyme hydrodynamically coupled to its environment increases significantly when undergoing changes in conformational fluctuations in a substrate concentration dependent manner, and is independent of the overall turnover rate of the underlying enzymatic reaction. Our results show that substrate-induced enhanced diffusion of enzyme molecules can be explained within an equilibrium picture and that the exothermicity of the catalyzed reaction is not a necessary condition for the observation of this phenomenon.



KEYWORDS: *Enzymes, catalysis, diffusion, conformational changes, hydrodynamic interactions*

In a quest for understanding fundamental molecular processes encountered in living systems, recent experimental progress led to the design, fabrication, and characterization of synthetic micro- and nanomachines relying on different propulsion mechanisms and the ability to reproduce functions inspired from molecular biology, such as cargo transport or chemical sensing.[1,2] Such autonomous objects could have major technological applications, provided that they are small enough and fully biocompatible. In this context, and going down in scale, enzyme molecules have received a lot of attention, as models of biological nanoscale transducers able to convert chemical energy into mechanical work. Biomolecules typically perform cyclic turnovers in which they bind to substrates and catalytically convert them to products while undergoing conformational changes.[3−6] Recently, in vitro studies of enzymes using fluorescence correlation spectroscopy (FCS) have revealed that their diffusion coefficient is enhanced in a substrate concentration dependent manner[7−10] and that the diffusion enhancement ΔD at substrate saturation was typically of the order of the bare diffusion coefficient of the enzyme D_0 measured in the absence of substrate molecules. This observation holds for a wide range of enzymes, which typically catalyze fast and exothermic chemical reactions with reaction enthalpies that can reach $40k_BT$ per molecule and catalytic rates up to $\sim 10^5$ s^{-1} for the particular case of catalase.[10,11]

This intriguing phenomenon, which could have major implications in the spatial organization of biological processes,[12] was subsequently investigated from a theoretical point of view. It was first suggested that the enhancement of the enzymes diffusion coefficient is directly proportional to the overall rate of the catalytic reaction and that there is a correlation between the degree of exothermicity of the overall reaction and the observed enhancement in diffusion.[10] In support of these findings, a theoretical scenario was proposed in which the energy released by the chemical reaction is assumed to be channeled into an asymmetric compression of the molecule and converted into a translational boost. However, the theoretical picture proposed in support of these experimental findings was subsequently criticized as it relies on an underestimate of the friction coefficient of the protein and on the hypothesis that the released energy is partitioned only over a small number of degrees of freedom.[13] Alternatively, we recently proposed that the exothermicity of the reaction catalyzed by the enzymes was responsible for collective heating of the sample container that could contribute to the enhanced diffusion of the enzyme molecules.[13]

The role played by stochastic swimming of enzyme molecules induced by conformational changes was also investigated within a nonequilibrium picture.[13−16] With a simplified description of the mechanochemical cycle of the enzyme, it was shown that the diffusion enhancement was

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controlled by the overall catalytic rate of the reaction k_{cat} through the relation $\Delta D \sim k_{cat}R^2$ where R is the hydrodynamic radius of the enzyme, and represents an upper bound for the typical length scale representing the magnitude of its conformational changes.[13] However, even for fast enzymes such as catalase, the relative change in the diffusion coefficient barely reaches the orders of magnitude observed in experiments.[13] It was finally proposed that enzymes could act as active force dipoles, that create nonthermal fluctuating solvent flows, and that could be responsible for enhanced diffusion.[17] In such a collective picture, the diffusion change is controlled by the volume fraction of enzymes in the sample, which is usually very small in the FCS experiments. Consequently, although such effects could potentially have important consequences for denser suspensions, they cannot account for the experimental realizations mentioned above.

Therefore, the status quo of the physical understanding of this phenomenon is that it is an intrinsically nonequilibrium process, and relatively satisfactory explanations were only proposed for enzymes that are sufficiently fast or catalyze sufficiently exothermic reactions. In search of a more complete physical picture, it is pertinent to probe whether exothermicity is a necessary condition for the phenomenon, and whether the enhanced diffusion is controlled by the overall catalytic rate. To this end, we experimentally studied aldolase, an enzyme involved in different fundamental metabolic processes such as glycolysis, because it has the following properties: First, this enzyme is known to be endothermic with a reaction enthalpy estimated ranging from 30 to 60 kJ/mol.[18,19] Second, the turnover rate of this enzyme is very low with a maximum of 5 product molecules generated per second at substrate saturation.[20] Aldolase converts its substrate fructose-1,6-bisphosphate (FBP) into the products dihydroxyacetone-phosphate (DHAP) and glyceraldehyde 3- phosphate (G3P).

Diffusion experiments were performed using fluorescent correlation spectroscopy (see Supporting Information) with samples containing 10 nM labeled aldolase in the presence of varied concentrations of fructose-1,6-bisphosphate (FBP, 0−1 mM). In the absence of substrate, the diffusion coefficient of aldolase molecules was $D_0 = 42.6 \pm 1.0\ \mu m^2 \cdot s^{-1}$. We show in Figure 1a the diffusion coefficient D as a function of the concentration of substrate. The diffusion coefficient of the aldolase molecules was found to increase in a substrate concentration dependent manner with relative enhancement that can reach up to 30%. In order to rule out the possibility of deagglomeration causing the enhanced diffusion of aldolase, we also compared the diffusion of aldolase before, during, and at the completion of the reaction. As shown in Figure 1b, while the diffusivity of aldolase increases during turnover, it returns to the base value after the substrate is consumed.

The observed enhanced diffusion of aldolase with similar relative magnitudes to the significantly faster enzymes and the same characteristic Michaelis−Menten dependence on the substrate concentration poses an apparent paradox: the enhanced diffusion cannot be controlled by the magnitude of the reaction rate but it exhibits the same dependence on the substrate concentration. Moreover, given the thermodynamic properties of aldolase, the nonequilibrium mechanisms relying on the exothermicity of the catalytic reaction cannot be extended to the present case. Therefore, our experimental observations lead us to reconsider the theoretical paradigm around this physical phenomenon. First, it is necessary to determine if this enhancement is due to an intrinsically

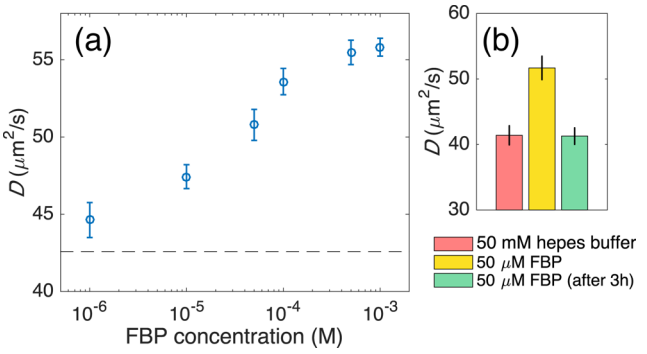

Figure 1. (a) Diffusion coefficient of aldolase molecules measured in experiments (the error bars represent standard deviations calculated for 15 different measurements under identical conditions) as a function of FBP (substrate) concentration. The dashed line corresponds to the base value of D in the absence of substrate. (b) The enhanced diffusion of aldolase in the presence of substrate returns to the base value (observed in the absence of the substrate) when the substrate is consumed. All values are significantly different with $p < 0.05$.

nonequilibrium process, or, in other words, if it is proportional to (or at least controlled by) the overall rate of catalysis. Second, we need to identify a mechanism that would provide quantitative answers to account for the observed order of magnitude for the diffusion enhancement.

The first step in our modeling consists in a careful analysis of the relevant time scales of the phenomenon. Our approach is motivated by recent studies of enzyme conformational changes[21,22] and in particular aldolase reaction pathways using fluorescence emission spectrophotometry,[23] which have revealed that the rates of conformational changes could be much higher than the actual chemical rate and reach values up to 10−100 s^{-1}. It is important to take account of how many competing time scales exist in the problem. The time scale for the actual conformational changes when they are triggered is of the order of the rotational diffusion time of the protein and is the shortest time scale in the system. The time scales for binding and unbinding of the substrate, which are purely physical processes at equilibrium since they do not involve subsequent conversion into products, are longer than the time scale for conformational changes but shorter than the time scale for chemical conversion. Because the overall catalytic reaction is much slower than the conformational fluctuations, it is reasonable to neglect the chemical step of the cycle altogether. Consequently, we assume that the protein exists in two different states, namely a free state and a bound state, in which a substrate molecule is present in the active site (see Figure 2a). Note that this simplified picture is an equilibrium description of the problem, which does not involve the chemical or catalytic step of the process and is therefore independent of the degree of exothermicity of the overall reaction.

In order to probe the importance of the catalytic step of the mechanochemical cycle, we have also measured diffusion of aldolase in the presence of pyrophosphate (PPi), which is a competitive inhibitor of aldolase and binds at the same active sites as FBP.[20,25] In the presence of PPi alone, diffusion of aldolase shows significant enhancement (Figure 3), demonstrating that the catalytic step of the reaction scheme is not necessary to lead to enhanced diffusion. These findings are consistent with recent experiments performed on citrate synthase and malate dehydrogenase, which suggest that the

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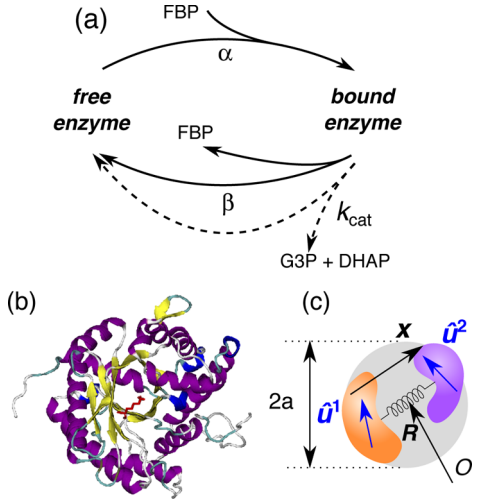

Figure 2. (a) Substrate binding and unbinding drives a stochastic two-state process. The enzyme switches randomly between two equilibrium states where it is either free or bound. (b) Structure of an aldolase monomer (Protein Data Bank ID: 1ADO, fructose 1,6-bisphosphate aldolase from rabbit muscle, subunit A), generated with visual molecular dynamics (VMD).[24] The residue colored in red indicates the location of the active site.[23] (c) Aldolase enzyme modeled as a dumbbell. ***R*** is the position of the center of mass of the enzyme, ***x*** represents its elongation. The gray sphere symbolizes the whole enzyme, whose typical size is denoted by *a*.

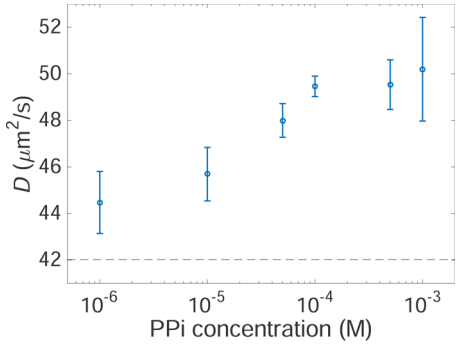

Figure 3. Diffusion of aldolase enhances with increasing pyrophosphate (PPi) concentration (the dashed line corresponds to the base value in the absence of inhibitor). PPi is a competitive inhibitor of aldolase.

diffusion coefficients of the enzymes are enhanced in the presence of their substrates even in the absence of their cofactors[12] and that binding/unbinding is sufficient to lead to enhanced diffusion of enzymes.

Relying on this simplified stochastic picture, we then aim to describe the effect of changes in conformational fluctuations induced by the binding and unbinding events. We first consider the simple case where the enzyme is always free (in the absence of substrate molecules). The state of the enzyme is then completely described by the position of its center of mass ***R*** and a vector **C**, which describes the conformation of the enzyme and whose dimension corresponds to the number of internal degrees of freedom. Given the complexity of the real structure of biomolecules (see Figure 2b for a representation of aldolase), **C** is a high-dimensional vector that does not need to be specified for now. The mobility coefficient μ of the enzyme depends on its geometrical properties, and therefore on its conformational state **C**. The overall diffusion coefficient of the enzyme as measured in the FCS experiments is an average on the conformations explored by the enzyme and can be related to the mobility through the fluctuation−dissipation theorem[26,27] as

$$D = k_B T \int d\mathbf{C} \mu(\mathbf{C}) p(\mathbf{C}) \equiv k_B T \langle \mu \rangle \tag{1}$$

where $p(\mathbf{C})$ is the probability to find the enzyme in a given conformation **C**. This expression is valid as long as the time scale on which conformational changes occur, which is comparable to the rotational diffusion time, is smaller than any other time scale.

In the presence of the substrate, the enzyme switches randomly between a free state and another state where it is bound to a substrate molecule. The binding rate α is expected to be proportional to the substrate concentration S. Noticing that the distribution of the conformation coordinate **C** is different in the two states (free and bound), we expect the rates α and β to depend on this coordinate. The detailed balance condition takes the form $\alpha\rho_f(\mathbf{C}) = \beta\rho_b(\mathbf{C})$, where ρ_f (respectively ρ_b) is the distribution of **C** knowing that the enzyme is in the free (respectively bound) state. Writing $\rho_f \propto e^{-U_f/k_BT}$ and $\rho_b \propto e^{-U_b/k_BT}$, where U_f and U_b are the effective potentials corresponding to given conformations, we get

$$\frac{\alpha}{\beta} \propto \frac{S}{K_0} e^{-[U_b(\mathbf{C}) - U_f(\mathbf{C})]/k_BT} \tag{2}$$

where K_0 is the bare equilibrium constant. The transitions of the enzyme between two equilibrium states therefore modify the effective distribution of the conformational variable. Assuming that the binding and unbinding rates α and β are very large compared to the intrinsic time scales of the enzyme, one can establish the effective distribution of **C** as

$$p(\mathbf{C}) \simeq \frac{1}{Z}\left[1 + \frac{S}{K_0} e^{-[U_b(\mathbf{C}) - U_f(\mathbf{C})]/k_BT}\right] e^{-U_f(\mathbf{C})/k_BT} \tag{3}$$

where Z is a normalization constant. It follows that the average of any conformation-dependent quantity $\Phi(\mathbf{C})$ can be written as

$$\langle \Phi \rangle = \langle \Phi \rangle_f + [\langle \Phi \rangle_b - \langle \Phi \rangle_f] \frac{S}{S + K} \tag{4}$$

where the averages $\langle \Phi \rangle_f$ and $\langle \Phi \rangle_b$ are defined using the corresponding Boltzmann weights e^{-U_f/k_BT} and e^{-U_b/k_BT}, and where we define the equilibrium constant $K = K_0 \frac{\int d\mathbf{C} e^{-U_f(\mathbf{C})/k_BT}}{\int d\mathbf{C} e^{-U_b(\mathbf{C})/k_BT}}$. Within this picture, the relative diffusion enhancement writes

$$\frac{\Delta D}{D_0} = \frac{\langle \mu \rangle_b - \langle \mu \rangle_f}{\langle \mu \rangle_f} \frac{S}{S + K} \equiv \mathcal{A} \frac{S}{S + K} \tag{5}$$

This result shows that even if the catalytic step of the chemical cycle is neglected in such a way that the modifications of the diffusion coefficient cannot be related to the rate of product formation, the relative change in diffusion still exhibits a Michaelis−Menten-like dependence over the substrate concentration, and is independent of the catalytic rate of the whole chemical reaction. The dimensionless coefficient $\mathcal{A}$ is a complex quantity, that depends on the shape of the interaction potentials $U_f(\mathbf{C})$ and $U_b(\mathbf{C})$, and that includes contributions from all the internal degrees of freedom of the enzyme that are affected by binding and unbinding. This simple eq 5, which

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contains the minimal ingredients of our new physical paradigm, can be used to fit the experimental data obtained for aldolase in the presence of the substrate FBP or in the presence of the competitive inhibitor PPi with $\mathcal{A}$ and K as free parameters. For the experiments with FBP (Figure 4a), we find $\mathcal{A} = 0.3$ and K

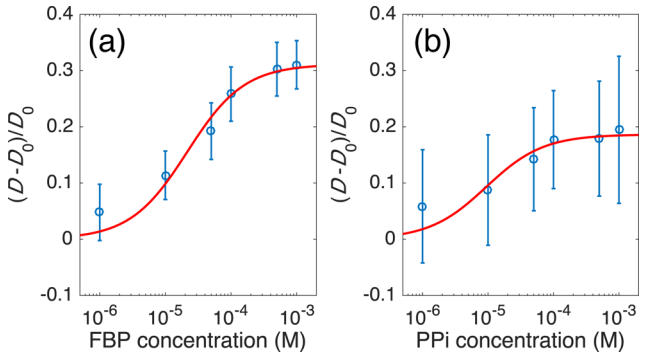

Figure 4. Relative increase of the diffusion coefficient of aldolase molecules measured in FCS experiments (symbols) in the presence of (a) FBP as substrate, (b) pyrophosphate (PPi) as competitive inhibitor, and compared to the fitting function $\frac{\Delta D}{D_0} = \mathcal{A}\frac{S}{K+S}$ (solid line), with $\mathcal{A}$ and K as free parameters. The relatively large error bars for the experiments performed with PPi originate from the error on the measurement of D_0, which affects the standard deviation for the quantity $\Delta D/D_0$.

$= 2.16 \times 10^{-5}$ M, which is comparable to the Michaelis constant reported for aldolase in the presence of FBP at physiological pH[20] (1.28×10^{-5} M). In the presence of the inhibitor PPi (Figure 4b), we find $\mathcal{A} = 0.19$ and $K = 9.4 \times 10^{-6}$ M, the dissociation constant given in the literature being 4.6×10^{-5} M.[28]

In order to get a more quantitative description of the changes in the averages mobility coefficients $\langle\mu\rangle_f$ and $\langle\mu\rangle_b$, we need to consider in greater details the modifications of the conformational fluctuations induced by binding and unbinding. The simplest way to describe the conformational state of the enzyme is to reduce the conformational state $\mathbf{C}$ to a single parameter R that describes the hydrodynamic radius of the enzyme. Structural studies of aldolase have recently shown that the effect of FBP binding was to bring residues near the active site closer one to another,[23] therefore effectively reducing the hydrodynamic radius of the molecule. The mobility coefficient of the molecule goes as the inverse of the hydrodynamic radius, so that the contribution to $\mathcal{A}$ coming from this effective size reduction can be estimated as $\mathcal{A}_1 \sim |\delta R|/R$, in a way that deformations of the order of a few Å can have a significant impact on the measured diffusion coefficient.

Then, in order to go further in the description of the internal degrees of freedom of the molecule and to take into account the effect of binding/unbinding on its elastic properties, we use a minimal dumbbell model (Figure 2c), where the structure of the enzyme is reduced to two hydrodynamically coupled subunits interacting via a harmonic potential, and which we recently studied in detail.[29] The conformational state $\mathbf{C}$ now reduces to a vector $\mathbf{x}$ that represents the elongation of the dumbbell. In the particular case where the dumbbell is symmetric, and in the limit where the subunits are far from one another, one can show that the averaged mobility coefficient is given by[29]

$$\langle\mu\rangle = \frac{1}{12\pi\eta}\left(\frac{1}{a_0} + \left\langle\frac{1}{x}\right\rangle\right) \tag{6}$$

where η is the viscosity of water, a_0 the typical size of the subunits and x is the length of the dumbbell. Binding of a substrate molecule to the enzyme will generally hinder the fluctuations of internal degrees of freedom, and therefore make the protein stiffer. The contribution $\mathcal{A}_2$ to the dimensionless coefficient $[\langle\mu\rangle_b - \langle\mu\rangle_f]/\langle\mu\rangle_f$ can be calculated explicitly by assuming that the potential energies associated with the internal variable x are of the form $U_f = \frac{1}{2}k_f(x-a)^2$ and $U_b = \frac{1}{2}k_b(x-a)^2$ with $k_b > k_f$ and where a is the typical size of the enzyme. In the limit of very large k_f and k_b with a finite difference $\delta k \equiv k_b - k_f$, we find $\mathcal{A}_2 \propto \frac{k_BT}{k_f a^2}\frac{\delta k}{k_f}$ up to a dimensionless prefactor of order 1. The dimensionless number $\frac{k_BT}{k_f a^2}$ represents the relative amplitude of the length fluctuations of the dumbbell and is bounded by unity, such that increased stiffness can significantly increase the enzyme diffusion coefficient. This contribution can be related to the concept of entropic allostery,[30] which suggests that ligand binding to a macromolecule can change its vibrational entropy, in addition to affecting its static structure.

Finally, this model can be refined by assuming that the subunits have more complex shapes and undergo orientational fluctuations (Figure 2c). The conformational state of the enzyme is then described by the vector $\mathbf{C} = (\mathbf{x}, \hat{\mathbf{u}}^1, \hat{\mathbf{u}}^2)$ where $\hat{\mathbf{u}}^1$ and $\hat{\mathbf{u}}^2$ are unit vectors characterizing the orientations of the subunits. These additional degrees of freedom will affect the overall diffusion coefficient of the dumbbell. We recently employed a Fokker–Planck description of the stochastic dynamics of the dumbbell and a careful treatment of the coupling between the internal and external degrees of freedom induced by hydrodynamic interactions to show that the internal fluctuations can contribute negatively to the effective diffusion coefficient of the position of the dumbbell.[29] It is beyond the scope of this paper to present the details of this calculation, and we simply give the following simplified and generic form for the effective diffusion coefficient

$$D = D_{ave} - \delta D_{fluc} \tag{7}$$

where the first term corresponds to the average contribution from the translational modes of the dumbbell, and the second term represents fluctuation–induced corrections arising from the internal degrees of freedom. The latter is controlled by the asymmetry of the dumbbell and the anisotropy of the individual subunits, and is typically a fraction of D_{ave}, depending on the precise geometrical properties of the dumbbell. Its negative sign is a generic feature of fluctuation–induced interactions.[31] In particular, this analysis indicates how hindering the orientational fluctuations of freely rotating parts of the molecule can enhance its overall diffusion. A more detailed theoretical study of this effect will be the object of a later publication.

Therefore, these contributions, that originate from a reduction of the hydrodynamic radius of the enzyme, an increased stiffness, or hindering of the internal modes of fluctuations of the enzyme can yield significant diffusion enhancements, which are of the order of a fraction of the bare diffusion coefficient of the enzyme. Although this extended dumbbell model is an idealized representation of the enzyme

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that greatly simplifies its structure, it contains, with very few internal degrees of freedom, the minimal ingredients to represent the compressional and orientational fluctuation modes that prevail inside a real macromolecule and should therefore accurately predict the main features observed with FCS experiments.

In summary, by employing aldolase, a slow enzyme that catalyzes an endothermic reaction, we demonstrated experimentally that exothermicity is not a necessary condition for the observation of enhanced diffusion in the presence of substrate molecules. These results challenge previous physical scenarios that were proposed to account for this phenomenon and that only held when the amount of heat released by the enzyme at each catalytic turnover was significant or when the overall catalytic rate was sufficiently large. Guided by these experimental results and by structural studies of aldolase, we proposed a new physical paradigm, in which the enzyme stochastically switches between two equilibrium states, in which it is either free or bound. Considering that binding and unbinding significantly affects the conformational fluctuations of the enzyme, we were able to measure the change in its diffusion coefficient as measured in FCS experiments in terms of its averaged mobility coefficients. Using simple physical arguments and a more subtle analysis of the fluctuation−induced effects mediated by hydrodynamic interactions, we generically show how substrate binding can modify the mobility and eventually enhance the diffusion of the enzyme.

Although we have obtained this result using the assumption that the binding and unbinding rates are considerably higher than the catalytic reaction rate, it is natural to expect that for faster enzymes these rates could be comparable in which case we will obtain a combination of the above effect and the stochastic swimming that is controlled by the (fast) reaction rate. This picture constitutes a new physical phenomenon, that was overlooked so far. Finally, we emphasize the generality of this mechanism: because substrate binding−unbinding is universal for enzymes, the proposed mechanism for enhanced diffusion should be universally present for all enzymes and should be observable provided the changes in the conformational fluctuations are sufficiently large in relative terms. While our main aim has been to propose a new generic physical mechanism, more detailed studies of the molecular structure of the enzymes, for example, using molecular dynamics simulation,[32] could help determine the precise characteristics that would allow enhanced diffusion of enzymes upon substrate binding and unbinding.

■ ASSOCIATED CONTENT

Supporting Information

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Details about the experimental methods and the FCS measurements (PDF)

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Notes

The authors declare no competing financial interest.

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