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 degrees of freedom,13 alternatively, we recently proposed that the enhanced diffusion of enzyme molecules in which they bind to substrates and catalytically convert them to products while undergoing conformational changes was 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 released energy 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 is 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.15–16 With a simplified description of the mechnochemical cycle of the enzyme, it was shown that the diffusion enhancement was
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.\textsuperscript{15} However, even for fast enzymes such as catalase, the relative change in the diffusion coefficient barely reaches the orders of magnitude observed in experiments.\textsuperscript{15} 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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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 $D_0 = 42.6 \pm 1.0 \text{ \mu m}^2 \text{s}^{-1}$. 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 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\textsuperscript{1,2,12} and in particular aldolase reaction pathways using fluorescence emission spectrophotometry,\textsuperscript{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.\textsuperscript{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
and where we defined the distribution of the conformational variable. Writing \( \rho_1 \propto e^{-U_f/k_BT} \) 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

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

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 \( \alpha \) and \( \beta \) are very large compared to the intrinsic time scales of the enzyme, one can establish the effective distribution of \( C \) as

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

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

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

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 \( K = K_f \frac{\alpha e^{-U_f(k_BT)}}{\alpha e^{-U_f(k_BT)}} \frac{e^{-U_b(k_BT)}}{e^{-U_b(k_BT)}} \).

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 \( U_f(C) \) and \( U_b(C) \), 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

**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: 1AJO, fructose 1,6-bisphosphate aldolase from rabbit muscle, subunit A), generated with visual molecular dynamics (VMD). The residue colored in red indicates the location of the active site. (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.
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 Pi with \( \mathcal{A} \) and \( K \) as free parameters. For the experiments with FBP (Figure 4a), we find \( \mathcal{A} = 0.3 \) and \( K \)

\[
\langle \mu \rangle = \frac{1}{12 \eta a_0} \left( \frac{1}{x} + \left( \frac{1}{x^2} \right) \right)
\]

where \( \eta \) 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 \( \mathcal{A}_1 \) to the dimensionless coefficient \( \langle (\mu)_b - (\mu)_i \rangle / \langle \mu \rangle \) can be calculated explicitly by assuming that the potential energies associated with the internal variable \( x \) are of the form \( U_i = \frac{1}{2} k_i (x - a)^2 \) and \( U_b = \frac{1}{2} k_b (x - a)^2 \) with \( k_b > k_i \) and where \( a \) is the typical size of the enzyme. In the limit of very large \( k_i \) and \( k_b \) with a finite difference \( \delta k \equiv k_b - k_i \), we find \( \mathcal{A}_1 \propto \frac{k_b^{\delta} k_i^{\epsilon}}{k_{b0}^{\delta} k_{i0}^{\epsilon}} \) up to a dimensionless prefactor of order 1. The dimensionless number \( \frac{k_b^{\delta} k_i^{\epsilon}}{k_{b0}^{\delta} k_{i0}^{\epsilon}} \) 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 \( \mathbf{C} = (\mathbf{x}, \mathbf{u}^1, \mathbf{u}^2) \) where \( \mathbf{u}^1 \) and \( \mathbf{u}^2 \) 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

\[
D = D_{\text{ave}} - \delta D_{\text{fluc}}
\]

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 \( D_{\text{ave}} \) 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
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 signifi-
cantly 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 fluctuations 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, 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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