Impulsive Enzymes: A New Force in Mechanobiology

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Abstract—We review studies that quantify newly discovered forces from single enzymatic reactions. These forces arise from the conversion of chemical energy to kinetic energy, which can be harnessed to direct diffusion of the enzyme up a concentration gradient of substrate, a novel phenomenon of molecular chemotaxis. When immobilized, enzymes can move fluid around them and perform directional pumping in microfluidic chambers. Because of the extensive array of enzymes in biological cells, we also develop three new hypotheses: that enzymatic self diffusion can assist in organizing signaling pathways in cells, can assist in pumping of fluid in cells, and can impose biologically significant forces on organelles, which will be manifested as stochastic motion not explained by thermal forces or myosin II. Such mechanochemical phenomena open up new directions in research in mechanobiology in which all enzymes, in addition to their primary function as catalysts for reactions, may have secondary functions as initiators of mechanosensitive transduction pathways.

Keywords—Cell mechanics, Mechanobiology, Myosin, Phosphorylation, Rheology, Diffusiophoresis, Molecular swimmer, Chemotaxis, Cytoplasm.

INTRODUCTION

Conversion of substrate to products by enzyme-mediated catalysis is a fundamental process in nature. For example, many biologically relevant enzymes convert ATP and GTP to their diphosphate forms, and harness the resulting energy to change the enzyme to a new conformation with a different function. For example, myosins,⁵² kinesins,⁵³ and dyneins³⁷ can

create power strokes by being in a tensed state stabilized by ATP and change conformation with the release of ADP and phosphate. This power stroke is then transmitted to the molecular motors' cargo.⁴² Such motor activity results in highly regulated and directional transport of the enzyme and cargo because these motors are specifically bound to polymer tracks, such as actin filaments and microtubules (reviewed in²¹). The forces (~10 pN) from these motors has been extensively and exquisitely measured.⁴⁷

Other enzymes in nature are not bound to fixed structures, but rather diffuse to and away from their substrates. Such enzymes are not expected to have directional transport and, until recently, were not expected to be force generating. For example, kinases of the mitogen activated protein kinase pathway catalyze the binding of a phosphate moiety to serine and threonine on a downstream kinase to convert it to an activated form.⁴³ The sequential phosphorylation of these kinases constitutes a biochemical pathway through which external stimuli can be converted to changes in transcription factor activation, DNA expression, and protein production. Peroxidases, another large family of enzymes, convert hydrogen peroxide to water and oxygen and are involved in disease resistance in plants and protection from reactive oxygen species (ROS) in animal cells. ROS are highly reactive oxygen radicals formed as a natural byproduct of oxygen metabolism and play a ubiquitous role in cell signaling related to atherosclerosis³⁶ and many cancers.³⁰ Enzymes such as catalase and many peroxidases can convert hydrogen peroxide into water, thus providing an important avenue for cellular protection from disease.

The cooperativity between diffusing enzymes in signaling pathways has been well documented, but the degree to which forces play a role in these processes has not been reported. Further, although it is reasonable that energy conversion during enzymatic reactions

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should produce a force, either through non-reciprocal conformation changes,^{7,16,17} or by phoretic mechanisms related to gradients arising from differences in diffusivities of reactants and products,^{6,27} to date there has been no direct experimental evidence for such a phenomenon. Through collaboration, our groups have recently shown enhanced diffusion of urease and catalase in the presence of their respective substrates, urea and hydrogen peroxide, and we have hypothesized that there exists a force per substrate turnover to explain this enhanced diffusion (see Fig. 1).^{35,44} Using Langevin/Brownian dynamics simulations, we determined that 12 and 9 pN forces per turnover were sufficient to cause the enhancement in diffusion of urease and catalase, respectively, as measured at the single molecule level using fluorescence correlation spectroscopy (FCS). Furthermore, forces from these enzymes are comparable to the forces produced by myosin, kinesin, and dynein motors and other molecular scale systems,^{32,33,57} and within the range to activate integrins,⁵⁵ biological adhesion molecules responsible for mechanosensation by cells, making force production

by catalysis a potentially novel mechanobiology-relevant event.

In this perspective article, we show that non-traditional enzymatic forces could be an important source of force in cells and yield mechanobiological processes related to stochastic pulsing, as well as convection of cytoplasmic fluid. Such research would need to obtain detailed direct measurements of this force, measure enhanced diffusivity with increasing substrate concentration, measure the rate of single molecule chemotaxis, and understand how this phenomenon could be used in cellular force generation, as well as the organization of soluble enzymes involved in sequential metabolic steps. Such cooperativity and organization could function to reduce the dimensionality of diffusion, usually reserved for membranes and filaments, and thus could accelerate certain signaling cascades. Research in this area has already established a new direction in mechanobiology and will lay the foundation for understanding the role of catalytic force generation in enzyme organization, transport, and mechanobiological function.

FIGURE 1. (a) Upon catalysis an enzyme takes a larger step than it would under simple Brownian (thermal) motion. (b) One possible mechanism of this large step is the electrical gradient that occurs when a small charged product (e.g., negative charge) diffuses faster than a larger charged product (e.g., positive charge). The resulting charge gradient could impart an equal and opposite force on the enzyme molecule the time of which is dictated by rotational and translational diffusion time.

Enzymes May Propel Themselves Through Asymmetric Generation of Products or Through Non-reciprocal Conformational Changes

Golestanian and colleagues reasoned that propulsion of single particles (e.g., enzymes) could occur in response to the generation of products in the vicinity of the particle.¹⁸ These products would create interfacial forces depending on osmotic gradients, charges, or other properties. Our group has calculated the electrophoretic forces locally upon reaction and estimated the force to be about 10 pN per reaction.³⁵ Furthermore, products need not be charged. It is possible that the existence of particles near a reaction center could affect diffusion simply by imposing a local particle concentration gradient. By solving the local diffusion coefficient when particles were produced asymmetrically, Golestanian found that the instantaneous velocity was $v = -k_B T / \eta * \lambda^2 / R * \rho$ where k_B is Boltzmann's constant, T is temperature, η is viscosity, R is the radius of the particle, and ρ is the solvent density. The value of λ depends on the type of phoretic mechanism. Phoresis could be due to local depletion of product particles near the enzyme surface and the resulting slip velocity (diffusiophoresis) or to the production of a spherical shell that is permeable to solvent but impermeable to product particles, resulting in radial flows of solvent (osmiophoresis). A significant caveat, however, is that the generation of local gradients depends also on the rotational diffusion time after which the force from local gradients will be randomized. The time scale of rotational diffusion is $T_r = 8\pi\eta R^3 / (k_B T)$. For $R = 2 \mu\text{m}$ the rotational correlation time is 50 s. If the propulsive velocity of $0.5 \mu\text{m/s}$, then the sphere can move $25 \mu\text{m}$ before its rotation causes loss of directional propulsion. For an enzyme that is 7 nm, the rotational correlation time is 1.9×10^{-6} s, at room temperature in water. Thus in less than $2 \mu\text{s}$ the enzyme will turn and lose any orientational information generated by products. Thus, there is evidence against the plausibility that self-propulsion of an enzyme is due to self-generation of products leading to electrophoresis, diffusiophoresis, or osmiophoresis.

Nevertheless, some researchers have suggested that self-propulsion by asymmetric production of products is feasible. Colberg *et al.* have reasoned that angstrom-sized molecules can propel themselves using products near the enzyme that interact with the enzyme *via* Lennard-Jones interaction potentials.⁵ Motion of solvent near larger motors on the 100–1000 s of nm can be described well using continuum theory. However, models of angstrom-size motors must consider that the motors are on the length scale of the solvent molecules they interact with. Thus the physics for particle–par-

ticle interaction dominates. In the Colberg study, the authors employed molecular dynamics of a simple model system consisting of a dimer motor in a solvent (dimer refers to having both catalytic and non catalytic sites). The dimer is assumed to produce spatially asymmetric catalytic activity and a resulting inhomogeneous distribution of products. This non-homogeneous distribution of products creates a concentration gradient that can yield propulsion, depending on features of the products and solvent. While their system did not include a structured solvent, such as water (they used argon), this system captured most of the features present in the experimental system described in Muddana *et al.* who experimentally measured enhanced diffusion at the angstrom-nm scale in response to catalytic activity.³⁵ The main results are that the dimer moves in the direction of its catalytic sphere; for small dimers, this effect is a smaller fraction of Brownian motion than it is for larger dimers. The reason for the propulsion is that the A and B product particles near the catalytic sight have different interaction potentials with the dimer (specifically the catalytic side) where the interaction potentials are Lennard-Jones potentials with an $1/R^6$ attractive component (where R is distance between molecular centers). In addition, because momentum must be conserved for both particles and solvent, fluid flows generated by catalysis play a role in propulsion. As an example, the authors found that for a large motor of 5.79 nm, the velocity of propulsion was 3.67 nm/ns. So for a time of 4.15 ns, the motor travels 15.23 nm, which is 2.63 times its length. This result would appear in the mean square displacement (MSD) vs. time as a ballistic motion for long times, as indicated in Fig. 6 of Reference 20. Thus, it is possible that self-generation of local concentration gradients by enzyme catalysis can lead to impulsive forces for each catalytic reaction.

Another self-electrophoretic example has been shown for nanoparticles that are near the size of single molecules (10 s of nm).²⁹ These authors created Janus particles on the order of 30 nm in diameter with Pt on one side and Au on the other side. Such particles can catalyze H_2O_2 on the Pt side yielding hydrogen ions and oxygen molecules, which can, in turn, be converted to H_2O on the gold side. This creation of flow of ions from the Pt to the Au side is balanced by a flow of ions along the particle surface toward the Pt side, thus propelling the motor towards the Pt side. Using dynamic light scattering, the investigators could identify the rotational relaxation time as well as enhanced diffusion. They found that these motors could have an impulsive swim of 0.66 mm/s (which is four orders of magnitude times the body length of the motor) before the rotation randomizes the motion.

As an alternative mechanism, it is possible that changes in conformation of the enzyme upon catalysis could lead to generation of a propulsive force, much like a swimmer moves water past him/her.¹⁷ At the ultralow Reynolds number regime of a molecule, if propulsion is to occur through conformational changes, then the net conformational change should be non-reciprocal. For a reciprocal conformational change (one in which the molecule reverts back to its original conformation) in a low Reynolds number regime, the molecule would return exactly to its original position, subject to rotational diffusion, according to the Scallop theorem.^{15,39} Conformational changes can occur in the microsecond to second time scale,^{13,22} thus a rotational correlation time of microseconds would suggest that rotation during conformational change in non-negligible. Molecules can swim only if they revert to a new conformation after the original conformational change, in other words, non-reciprocal conformational changes. However, even this mechanism of propulsion would be randomized by rotational diffusion.

A possible method to bridge atomistic simulations with continuum is the mesoscopic approach by Cressman and colleagues.⁷ In this approach, the authors use the mesoscopic strategy of describing a molecule as an elastic network and a particle-based description of the solvent interactions defined by Lennard-Jones potentials and multiparticle collisional dynamics. In this study, the authors coupled the catalytic but non-reciprocal conformational changes occurring after catalysis to the local fluid to yield self propulsion of the molecular swimmer.⁴¹ In this case the binding of a molecule such as ATP, which provides energy to the system, causes a conformational change in the enzyme, which is described as a perturbation in the free energy of the molecule. This system then converts to an intermediate, new conformational state, and finally reverts back to its original state after losing ATP. Since this molecule is in a solvent, motion of the molecule leads to flows of the solvent. In other words, the energetic forward path to get to a conformational change is often different than the energetic reverse path to return to the original state. These non-reciprocal forward and reverse alterations in the interaction energies between the various components of the molecule lead to a swimming propulsion, because of the associated fluid flows around the molecule.

We have explored experimentally some of the potential mechanisms of enhanced diffusion of urease including local pH changes and self electrophoresis.³⁵ Using SNARF-1 tagged to the enzyme and calibrating the fluorescence lifetime of SNARF-1 with pH we detected significant increase in the local pH from 7 to 8.2 (increased local alkalinity from production of

OH⁻ ions) upon reaction. However, the mechanism of pH-induced enhanced diffusion, which was theorized to be through increases in protein-protein and protein counter-ion columbic interactions, was not sufficient to account for the increase in diffusion of urease. Furthermore, we calculated the expected force due to self-electrophoresis. In this mechanism, reactions produce a large ionic species, which diffuses away from the enzyme a short distance, and a small counter ion, which diffuses a larger distance. As a consequence, an instantaneous electric field is generated which can lead to the generation of a force on the enzyme (see Fig. 1b). However, as mentioned earlier, the rotational diffusion of small enzymes are sufficient to randomize the orientation of the molecule before an electrical gradient can be generated, suggesting that, while electrophoretic mechanism can be calculated to provide sufficient force, this force may never be realized by the enzyme in a directed fashion. Furthermore, the local heat generated by the enzyme seems unlikely to account for propulsion since the increase was in the range of microkelvin. Thus the precise mechanism of enzyme propulsion remains unknown.

Enzymatic Forces can be Estimated Using Enhanced Diffusion and Langevin Dynamics Simulations

We have recently discovered that enzymatic reactions can induce increases in the diffusion coefficient of three different enzymes, catalase, urease, and DNA polymerase, in the presence of their respective substrates, hydrogen peroxide, urea and ATP.^{35,44,45} In those studies, we used FCS to measure the diffusion coefficient of a fluorescently tagged enzyme. FCS is an ultrasensitive technique that can detect the motion of individual fluorescent molecules diffusing into and out of a diffraction-limited observation volume (1–2 fL).^{10,31} A time-correlated single-photon counter (TCSPC) was incorporated into the instrument to measure the fluctuations in fluorescence intensity within the observation volume, arising from diffusion of the analyte molecules across the observation volume boundary.¹⁹ The fluctuating fluorescence signal was then autocorrelated and fit by a theoretical 3D diffusion model to determine the enzyme diffusion coefficient, D , according to:

$$G(\tau) = \frac{1}{N} \left[1 + \left(\frac{\tau}{\tau_D} \right) \right]^{-1} \left[1 + \left(\frac{1}{w^2} \frac{\tau}{\tau_D} \right) \right]^{-1/2} \text{ with } \tau_D = \frac{r^2}{4D}, \quad (1)$$

where τ_D is the characteristic diffusion time across the laser beam waist of radius r and height w . $G(\tau)$ is the autocorrelation function, N is the number of molecules

on average in the confocal volume, and τ is the arrival time of photons to the detector.

We estimated the force responsible for the enhanced diffusion using Langevin dynamics simulations. Modeling was conducted by providing an impulse force, F , the timing of which corresponded to the reaction rate. The movement of the enzyme in r was inhibited by a drag coefficient, γ , according to

$$\frac{dr}{dt} = \frac{1}{\gamma} F(r) + \eta(t), \quad (2)$$

where $\eta(t)$ is the stochastic force with

$$\langle \eta_i(t) \cdot \eta_j(t + \Delta t) \rangle \geq 2\Delta t \delta_{ij} k_B \frac{T}{\gamma}, \quad (3)$$

where δ_{ij} is the Kronecher delta. Equation (2) was numerically integrated using

$$r(t + \Delta t) = r(t) + \frac{\Delta t}{\gamma} F(t) + \left(\sqrt{2k_B T \frac{\Delta t}{\gamma}} \right) r^G, \quad (4)$$

where γ is the Stokes–Einstein drag coefficient for a spherical particle, k_B is Boltzmann’s constant, r^G is the Gaussian distributed set of random numbers with mean = 0 and standard deviation = 1, and Δt is the integration time step (10 ns).

As an example, the presence of hydrogen peroxide in the solution caused a significant increase in the diffusion of catalase molecules. Catalase’s measured diffusion coefficient increased from $6.0 \times 10^{-7} \text{ cm}^2/\text{s}$ in water to $8.8 \times 10^{-7} \text{ cm}^2/\text{s}$ in 0.1 M hydrogen peroxide solution, i.e. an increase of ~45% (Fig. 2a).⁴⁴ Moreover, the diffusion coefficient of the enzyme increased in a substrate concentration-dependent manner. To

ensure that the enhanced enzyme diffusion was caused by substrate turnover, the diffusion coefficient of catalase inhibited by sodium cyanide⁴ was measured in the presence of substrate, and no significant effect of substrate concentration on the enzyme diffusion coefficient was observed.

The force due to a single catalytic turnover event for a catalase enzyme was estimated as follows: Given a maximum catalytic rate of $2.12 \times 10^5 \text{ s}^{-1}$ (for each catalytic site of catalase),⁴⁸ an artificial force can be applied for 10 ns on the particle in a random direction every time a reaction takes place (i.e., every 1.2 μs). The simulation trajectories were obtained for a total of 60 particles, for a total simulation time of 100 ms for each particle. Diffusion coefficients were computed from the slope of the mean-square displacement curve at long time scales. A range of forces (1–20 pN) was screened to identify the force required for the experimentally observed increase in diffusion coefficient. By adjusting the force per reaction in successive Langevin dynamics simulations until the simulated diffusion coefficient matched the experimental one (Fig. 2b), the force per reaction turnover was estimated to be 9 pN/turnover. This is well within the range of the forces measured for other molecular scale systems, including polymerases and motor proteins.^{32,33,57}

Forces are Sufficient for Molecular Chemotaxis

It has been theorized that if a diffuser experiences a diffusivity gradient, it can move up that gradient and experience directed motion.¹¹ Velegol *et al.* explored this phenomenon by observing the chemotaxis of non-biological colloidal rods. In this system H_2O_2 was the

FIGURE 2. (a) Increase in catalase diffusion was attenuated by catalase inhibitor, sodium cyanide. ($n = 10$, $P > 0.01$). (b) Brownian dynamics simulations predict 9 pN per turnover for 50% increase in diffusion. Figure adapted from.⁴⁴

“attractant” and the Pt–Au bi-metallic rods were the devices that were chemotaxing.²⁵ In this experiment, an H₂O₂ soaked agarose gel was placed at the center of a chamber filled with the bimetallic rods. The rods moved toward the gel and accumulated there after a time of 110 h. While a long time, the size of these rods means that their diffusion was slow. Nevertheless the accumulation over time indicates that they moved up a substrate concentration gradient. In fact, their speed was about 0.6 $\mu\text{m/s}$ near the gel and 0.1 $\mu\text{m/s}$ about 1.5 mm away from the gel. As the concentration gradient decreased over time, the chemotactic velocities were also reduced. The mechanism for this chemotaxis was active diffusion accomplished by random stochastic diffusion with an additional electrokinetic translation. It is believed that when a rod moves up a gradient, it experiences a new concentration. At that location it has a higher diffusion coefficient and moves even further. When a rod moves down a concentration gradient, it moves less. The net result is that rods move towards locations of increasing concentration of substrate.

In a similar manner, we reasoned that it might be possible for single molecules to “chemotax” (Fig. 3a), where “chemotaxis” is defined by the general movement of enzymes towards areas of higher substrate concentration. The scheme corresponds to a two-inlet microfluidic system, designed to allow enzymes and substrate to flow through different inlets, generating a sustained substrate concentration gradient along the interface. As we have shown, the diffusivity of active enzyme molecules increases with the increase in local substrate concentration. Following this assertion, if at any time, N enzyme molecules are considered to be present along the interface, substrate turnover will allow one fourth of them to move up the gradient—since they have equal probability of moving in any direction from the point of reaction. If the reaction-induced impulses last for a time interval, on average $N/4$ molecules will move into the substrate channel in the first time interval, covering a certain distance dictated by their hydrodynamic radii and solution viscosity. In the next time interval, a total of $N/16$ molecules will move further up the substrate channel based on the same principle. However on this occasion, the presence of substrate will cause enzymes to cover a larger distance compared to what they could sample in pure buffer. The excess migration results in chemotactic behavior of active enzymes and their collective direction migration towards higher substrate regions. The fractions of enzyme molecules moving within the substrate channel will however decrease continuously as the molecules move towards an area where the substrate concentration gradient is less steep.

To experimentally explore the idea of molecular chemotaxis we have shown that other enzymes besides urease have enhanced diffusion in the presence of substrate, signifying that this may be a broadly applicable phenomenon.⁴⁴ We hypothesized that when a substrate concentration gradient exists, enzymes chemotax up it. Further, when two enzymes are in juxtaposition with each other and the product of one is the substrate for the other, the enzymes can be drawn together, presumably because the local concentration gradient of substrate provided by the donor molecule for the acceptor molecule. To test these ideas, an H₂O₂ gradient was imposed in a two inlet, one outlet microfluidic channel by flowing in catalase in one channel and H₂O₂ in the other. This setup caused the catalase to migrate toward the H₂O₂ only when the substrate was present and the enzyme was active (Fig. 3c). When, instead, glucose oxidase and glucose (whose product is H₂O₂) were introduced in one inlet and catalase was introduced to the other inlet, catalase migrated toward the glucose oxidase side, suggesting an H₂O₂ gradient existed from glucose oxidase activity, sufficient for the molecular chemotaxis of catalase. We further created a finite element simulation of the convection–diffusion equation (Eq. 5) in which the relationship between catalase diffusion coefficient and H₂O₂ concentration was determined by FCS and input into the simulation as a substrate concentration-dependent (variable) diffusion coefficient (Eq. 6; Fig. 4).

$$\frac{\partial c}{\partial t} = \nabla \cdot (D_c \nabla c) - \nabla \cdot (\vec{v}c) + R, \quad (5)$$

$$\text{with } D_c(\text{m}^2/\text{s}) = 31.8 \times 10^{-11} + 9.03 \times 10^{-12} * \frac{\sqrt{c}}{0.65 + \sqrt{c}}, \quad (6)$$

where D_c is the diffusion coefficient of catalase that depends on the concentration (mol/m^3) of its substrate [H₂O₂]. The relationship between D_c and [H₂O₂] was solved by fitting an empirical model to the data obtained by FCS. Molecular chemotaxis was predicted that was equivalent to experimentally observed values.

It is well known that enzymatic motors participate in DNA synthesis and vesicular transport in a highly regulated fashion.^{1,12} Our group has probed the fundamental question of whether non-bound, non-traditional motors exist that create forces simply by catalyzing enzymes. In this case, a single enzyme can generate sufficient force to cause its own movement. Since the rate of force impulses depends on the substrate concentration, we have shown that the movement can become directional through the imposition of a gradient in substrate concentration, a situation that

FIGURE 3. (a) Schematic of molecular chemotaxis. The net effect of diffusion coefficients that depend on substrate concentration is that, on average, enzymes in the presence of a substrate diffuse more than ones in the absence of substrate (see text). (b) Schematic representation of Y-shaped microfluidic channel used for enzyme chemotaxis studies. (c) Plot of mean normalized ('1' corresponds to maximum fluorescence intensity and '0' corresponds to minimum fluorescence intensity) fluorescence intensity profile as a function of lateral position along the width of the channel (numbers correspond to x-position on graph) showed a shift for catalase (enzyme side) towards 10 mM hydrogen peroxide (red line; maximum standard deviation of 0.039 a.u. (arbitrary units), as compared to water (blue; maximum standard deviation of 0.003 a.u.), when viewed $\sim 400 \mu\text{m}$ from the entrance of the channel (location indicated by vertical blue line). Figure adapted from.⁴⁴

parallels the chemotaxis of whole cells. This novel demonstration and harnessing of enzymatic conversion of chemical energy to mechanical forces at the nano-scale vastly expands the available sources of force in cells that can be used for directed molecular diffusion.

Enzymatic Forces are Sufficient to Pump Fluid

Since enzymes have been shown to exert force, this force should be able to be transferred to the surrounding fluid.⁴⁶ In this scenario, immobilized en-

FIGURE 4. (a) Velocity profile from FEM model. Max velocity (red) = 10^{-3} m/s. (b) Concentration profile of substrate (H_2O_2). Red is max concentration of substrate. (c) Proposed modification to collect active enzyme that has migrated to upper part of channel.

zymes could pump fluid past themselves. While it would be difficult to coordinate the timing and direction of pulsing, in a closed system, the creation of local force on solvent induced by enzymes would cause movement of the fluid. Local movement of fluid (and changes in density) would cause convective currents in the device. This idea has been realized in a recent study.⁴⁶ In the case of catalase, lipase, and glucose oxidase that were immobilized over a surface inside a secure, sealed hybridization chamber by thiol-gold interaction, enzymatic reactions at the bottom surface of the chamber resulted in a stovepipe effect where local decreases in density caused fluid to rise above the gold-enzyme surface (Fig. 5). This resulted in inward flow near the gold surface and outward flow above. Interestingly, urease induced flow in the opposite direction with outward flow at the bottom surface and inward flow at the top. Thus it is possible that urease causes an increase in local fluid density resulting in a sinking of the fluid towards the gold surface. In all cases, the pumping velocity was proportional to the reaction rate, suggesting that pumping was caused by enzymatic turnover of substrate. In this study it may be possible to rule out some mechanisms of pumping. Mechanisms that rely on the properties of the products, such as diffusiophoresis, osmiophoresis, and self-electrophoresis are unlikely to explain the pumping. Each of these mechanisms should be independent of the orientation of the pump. In this study, when the chamber was inverted, the pumping direction was reversed. This reversal suggests that local density gradients induced by the reactants cause the movement of the fluid around them. The density theory makes uni-

versal the generation of local forces by any reaction, irrespective of the products. But what is the mechanism of local density changes? Many reactions are exothermic. Therefore a local increase in temperature should reduce the local density and induce fluid flow. However, for urease, exothermic considerations suggest the local density should decrease, but the pumping direction suggested that fluid densities near the reaction sites increased. Urease causes the conversion of urease into the charged products ammonium and bicarbonate ions. These charged products would locally cause an increase in density of the fluid, which appears sufficient to counteract the decrease in water density from local increases in temperature.

Importantly, this study continues the previous ones by exploring the enhanced diffusion of DNA polymerase.⁴⁵ Aside from the obvious importance of this enzyme biologically, the study demonstrates that enzymes that use ATP (e.g., almost all cell-relevant kinases), may be force generating. Regarding mechanism, it is unlikely that temperature-induced changes in local density are responsible for enhanced diffusive motion of DNA polymerase because the increase in temperature was estimated to be in the microkelvin range. Similarly, the influence of charge was found to be negligible. It is possible that DNA polymerase undergoes a series of conformational changes with substrate turnover, and that each conformational change induces a movement of local solvent leading to a kick force and thus enhanced diffusion. However, pumping experiments performed in this study suggest that local density fluctuations, induced either through direct coupling of non-reciprocal conformational

FIGURE 5. (a) Gold is patterned onto a glass surface and a self assembled monolayer (SAM) of thiols are place on the gold. Enzymes are attached to the thiols. Enzymatic reactions create convective flow in the device, which can be revealed with (b) tracer particles in the solution. Figure adapted from.⁴⁶

change in enzyme to solvent, or through the conversion from dATP to dAMP + PPi could induce a local decrease in solvent density.

Non-traditional Enzymatic Forces May Drive Stochastic Oscillations in Cells and Movement of Cytoplasmic Fluid

We conclude by considering the possible role of enzymatic forces in the generation of stochastic motions in cells and accompanying enhanced diffusion and convection of cytoplasmic fluid. An important recent study that captures some of these elements was described by Guo and colleagues.²⁰ In this study, the authors probed the origins of super-diffusive behavior of particles injected into cells. It has long been appreciated that the cytoplasm of living cells is highly dynamic.^{8,23,24} Much of the dynamism originates from molecular motors that are attached to tracks or filaments on which the motors carry cargo in a directed, processive fashion. Dynein, kinesins, and myosins follow this model. Furthermore, coordinated conformational changes of proteins such as myosin II can yield directional forces that are responsible for cell migration, breakdown of cell-cell junctions, contraction of the cell, and the apparent stochastic motion of the cell cytoplasm. It is now known that the motion of particles in cells is not entirely diffusive.^{54,56} If it were, the MSD of these particles as a function of time would

be linear. However, this relationship is highly non-linear owing partly to the viscous and elastic nature of the cytoplasm. But, importantly, the non-linear relationship between MSD and time is also because of active motions of the cytoplasm caused by motors. To a large degree, myosin II was the motor causing super-diffusive behavior, as inhibition of this motor by blebbistatin reduced the slope of the MSD vs. t graph to a value closer to that expected from purely thermally driven forces. However, not all of the deviation could be attributed to myosin II. In this respect, the recent study by Guo represents a turning point in understanding cellular rheology and force generation. A key observation relevant to the force generation by enzymes is "... when [the authors] depleted cells of ATP through addition of 2 mM sodium azide and 10 mM 2-deoxyglucose, [they] observed a force spectrum that is consistent with purely thermal fluctuations over the full frequency range for the viscoelastic medium measured directly for these cells... This suggested that, while actomyosin contractions are a significant source of intracellular forces, other enzymatic activities also contribute to the forces and hence the motion experienced by intracellular objects." In other words, inhibition of myosin II reduced a portion of the stochastic motion of cytoplasmic tracers that was above the stochastic motion explainable by thermal forces, but only after ATP was depleted was the entire super-diffusive regime abolished. Such a change was

also shown in an early report from Hoffman and colleagues who showed that ATP depletion reduced stochastic motion of tracer particles in at least some frequency ranges.²³ In that report, the investigators did not attempt to distinguish between ATP-driven motion in general and myosin II in particular. This observation that ATP depletion is needed to abrogate super-diffusive motion of tracer particles beyond that explainable by myosin II activity, suggests that, while myosin contraction of actin filaments is one force responsible for motions of the cytoplasm, there are other origins of stochastic fluctuations in cells that are ATP dependent. We suggest that enzyme-derived impulsive forces provide a source of these fluctuations. In fact, most of the super-diffusive behavior was abolished by incubation with ATP-depleted media, suggesting the types of enzymes responsible for this super diffusive behavior are ATP-driven. The myriad of kinases in cells fit this model very well. Indeed, DNA polymerase, which is ATP dependent, has been shown to deliver impulsive forces that result in enhanced diffusion on the same order of magnitude as urease and catalase.⁴⁷ The frequency range of cellular stochastic fluctuations is from 10^{-1} to 10^2 s⁻¹ (these are the ones measured in²⁰), which is in the range of reaction rates for many enzymes. In the Guo study, the authors used a model to estimate the density of myosin of $1/\mu\text{m}^3$ along with an impulse force of 10 pN to account for the observed fluctuations of tracers observed in their study. If the origin of these non-myosin II force fluctuations could be identified, it would be interesting to carry out this calculation for the remaining enzymes, each exerting a 10 pN force on organelles or filaments.

One important distinction is that not all enzymes are anchored to organelles. Non-anchored enzymes are still force generating, however, and could potentially induce local fluid flow through the cytoplasm by stirring liquid and directing it to various parts of the cell. This idea was explored in²⁰ (Fig. 7) using the diffusive spread of a photo-activatable water soluble dye, Dendra2, injected into the cytoplasm of normal and ATP depleted cells. Depletion of ATP significantly reduced the rate at which the photo-converted dye was transported throughout the cell, suggesting that ATP-dependent motors facilitated convective-diffusive movement of the liquid component of the cytoplasm, in addition to its observed role of tugging stochastically on the cytoskeleton.

Future Directions in Mechanobiology: Harnessing Stochastic Forces by Cellular Enzymes

Future work on this phenomenon of catalysis-enhanced enzyme diffusion will need to attend to three

research areas. First, there remains a fundamental need to determine the mechanism(s) of enhanced diffusion at the single catalytic event level. Second, there will be a need to determine whether these forces do, indeed, result in biologically relevant events, or whether they are simply a byproduct of catalysis that cells simply ignore. Third, from an engineering point of view, it will be interesting to demonstrate whether useful work can be gained from these processes to drive engineering of lab-on-a-chip applications not possible with current technologies, or whether these phenomenon could be scaled up to large scale chemical separations based on catalytic activity.

What is the Mechanism of Catalytic Force and is it Generalizable?

Despite the increase number of publications on catalysis-enhanced diffusion, there remains uncertainty as to the origin of the impulsive force, and whether there is a common origin at all. Our data suggests that diffusion-vs.-substrate concentration follows the general form of Michaelis–Menton kinetics, but differs quantitatively, suggesting that, while increased diffusion may result in part from reaction rates, the nature of the products (size, charge, etc.) may also be important. In a recent paper by Reidel *et al.* it was reasoned that heat from exothermic reactions is channeled through the protein structure and creates an acoustic wave near the protein-solvent interface.⁴⁰ If the reaction center was not at the center of mass of the enzyme, this acoustic wave would create an impulsive force. If this is true, then this acoustic wave should be measureable using methods used in photoacoustics. For the enzymes tested, there seems to be a correlation between exothermicity and enhanced diffusion, supporting this idea as a universal mechanism. Interestingly, the group estimated the force due to acoustic waves to be about 500 pN/nm² which, for a 4 nm radius particle, results in a 10 pN force per reaction, almost exactly what was predicted using Langevin dynamics simulations of urease³⁵ and catalase.⁴⁴ Therefore, heat-induced acoustic waves remains a plausible, and generalizable mechanism for enhanced diffusion. However, it is possible that endothermic enzymes also exhibit enhanced diffusion (unpublished observations) suggesting that other mechanisms besides heat induce forces.

To test the hypothesis that internal heat generation results in an acoustic wave that drives diffusion would require methods similar to those used by Muddana *et al.* involving environmentally sensitive dyes analyzed with time resolved fluorescence.^{20,35} The authors hypothesized that local pH changes might be responsible for the enhancement of diffusion of urease. They

tagged the enzyme with a pH sensitive dye, SNARF-1, which could directly record the local pH on a per molecule basis. However, although pH did change with reaction, it did not scale sufficiently with reaction rate to explain the increased diffusion. Nevertheless, such measurements are likely only possible with TCSPC instruments, which can simultaneously determine fluorescence lifetime (which is a concentration-independent measurement of local molecular environmental variables) and FCS, which can determine the impulse-dependent enhancement of diffusion.

Do these Forces Result in Biologically Relevant Events?

We and others have determined that catalytic reactions were on the order of 10 pN of forces, which are biologically relevant.^{35,40} For diffusive enzymes it is difficult to envision that such forces would yield useful work (force times distance) in a particular direction, in contrast to enzymes (e.g., kinesins) anchored on filaments. However, it is possible that cells can capitalize on the increased stirring that yields something akin to cytoplasmic streaming, which has been noted in eukaryotic cells.¹⁴ Second, if the enzymes are bound to membranes, they could yield significant dynamics bending events which have been shown to be important for organization of signaling complexes in membrane (e.g., curvature, etc.).^{50,51} Third, many enzymes are connected to adaptor proteins and could yield mechanobiologically relevant force on these complexes.²⁸ As such, catalytically generated stochastic forces may need to be considered as background forces that the cell either has to deal with (organize proteins in order to resist these forces) or harness them for useful work. Finally, the area of glassy rheology is a very important concept in cell mechanics.^{2,38} It may be possible that these stochastic forces are a source of such rheology, which is critical for functions such as endothelial cell shear sensing,⁹ migration,² and bacterial rheology.³⁸

Can Useful Work can be Gained from these Forces?

Finally, it may be possible to use such forces for biological separation in lab-on-a chip applications. While the dependence on diffusion may make large scale separation impractical, it is still possible to do this in a chip, where large volumes are not necessary. The aim of such a project would be to develop microfluidic technology capable of harnessing newly discovered force generation by single enzyme molecules for useful separation of active enzymes from their inactive counterparts.⁹ An example of a diagnostic target might be the hydrolase enzyme, influenza neuraminidase (an established biomarker for the flu virus). Since the enzyme influenza neuraminidase plays a key

role in the virulence of the flu virus,³⁴ the ability to separate, concentrate, and identify trace levels of this biomarker from macroscopic quantities of body fluids should allow the detection of the flu virus in a person within the first 24–48 h of infection. Detection during the first 2 days after infection is critical for effective antiviral treatments,³ but current point-of-care diagnostics devices are not sufficiently sensitive to easily detect these early stages of infection.^{26,49} We believe that chemotaxis-based separation will enable concentration of active enzymes for enhanced detection sensitivity.

CONCLUSION

We propose that non-traditional ATP-dependent enzymes provide sufficient force for the stochastic motion of the cytoplasm, the organization of signaling complexes, and the convective transport of fluid in cells. While most of the work to date in this area has been on enzymes that are not widely studied in cells, the focus on cellular enzymes, particularly ones that catalyze the hydrolysis of ATP or GTP, would uncover a source of energy and force in cells that may be biologically relevant. Thus, the mechanical impulses generated by enzymatic turnover of substrate may be a new force for cells, and scientists, to reckon with.

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CONFLICT OF INTEREST

Peter J. Butler, Krishna K. Dey, and Ayusman Sen have no conflicts of interest to declare.

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