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# Stochastic pumping of non-equilibrium steady-states: how molecules adapt to a fluctuating environment

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In the absence of input energy, a chemical reaction in a closed system ineluctably relaxes toward an equilibrium state governed by a Boltzmann distribution. The addition of a catalyst to the system provides a way for more rapid equilibration toward this distribution, but the catalyst can never, in and of itself, drive the system away from equilibrium. In the presence of external fluctuations, however, a macromolecular catalyst (e.g., an enzyme) can absorb energy and drive the formation of a steady state between reactant and product that is not determined solely by their relative energies. Due to the ubiquity of non-equilibrium steady states in living systems, the development of a theory for the effects of external fluctuations on chemical systems has been a longstanding focus of non-equilibrium thermodynamics. The theory of stochastic pumping has provided insight into how a non-equilibrium steady-state can be formed and maintained in the presence of dissipation and kinetic asymmetry. This effort has been greatly enhanced by a confluence of experimental and theoretical work on synthetic molecular machines designed explicitly to harness external energy to drive non-equilibrium transport and self-assembly.

## 1. Introduction

Molecular systems such as enzymes, biomolecular motors, receptors, *etc.* carry out their functions in fluctuating environments. The fluctuations arise not only because of the ineluctable

presence of thermal noise by which chemical transformations, including conformational changes, occur but also possibly through externally driven perturbations by which energy is input from the environment. Examples include external fluctuating but zero-average electric fields that were shown to be able to drive uphill motion of solute molecules across a membrane and externally maintained oscillations of the redox potential that can drive a synthetic molecular pump. In the mid 1980's a theory, then known as electro-conformational coupling,<sup>1–3</sup> and now more generally termed stochastic pumping,<sup>4–7</sup> was developed to describe the mechanism by which energy from externally driven fluctuations is harvested by macromolecules and used to do work on the environment. The theory showed that an enzyme catalyzing a reaction  $S \rightleftharpoons P$  could absorb electrical work through conformational transitions involving charge motion and use part of the absorbed energy to drive the formation and maintenance of a non-equilibrium steady state between substrate and product given by<sup>8</sup>

$$\frac{[P]}{[S]}_{ss} = K_{eq} \left\langle e^{\Delta\theta[\delta F(t') - \delta F(t'')]} \right\rangle \quad (1)$$

where  $K_{eq}$  is the standard equilibrium constant between substrate and product. Here and elsewhere in this paper energies are given in units of the thermal energy  $k_B T$ . The term  $\langle e^{\Delta\theta[\delta F(t') - \delta F(t'')]} \rangle$  is the weighted time average of the exponential of the work exchanged between the source of the fluctuations



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and the enzyme in the conversion of S to P. The quantity  $t'$  is the time at which S binds and  $t''$  is the time at which P dissociates in a specific trajectory. The term  $\delta F(t)$  is the time dependent deviation of a generalized thermodynamic force (pressure, temperature, electric field, chemical potential, *etc.*) from some standard value. The generalized thermodynamic force,  $F$ , is an extensive parameter. The canonically conjugate generalized thermodynamic displacement  $\Delta\theta$  is the difference of an intensive parameter between the free and bound state of the enzyme and is a characteristic of the enzyme. The equilibrium constant  $K_{\text{eq}}$  is of course a state function and depends only on the thermodynamic properties of S and P. On the other hand, the average  $\langle e^{\Delta\theta[\delta F(t') - \delta F(t'')]} \rangle$  depends on the enzyme and on all possible paths (*i.e.*, on times  $t'$  and  $t''$ ) by which S is converted to P by the enzyme. This exponential average can be greater than, less than, or equal to one depending on the kinetic details of the enzyme conformational changes, and hence the term  $\langle e^{\Delta\theta[\delta F(t') - \delta F(t'')]} \rangle$  can favor substrate or product relative to the equilibrium case.

To illustrate the relationship between the enzyme dynamics and the thermodynamics of the catalyzed reaction consider the reaction  $S \rightleftharpoons P$ . This reaction will inexorably proceed, albeit slowly, toward equilibrium. If an external driving  $\delta F(t)$  that does not influence the reaction itself is applied once the system has reached equilibrium nothing will happen. Alternatively, if an enzyme is added, the reaction will proceed toward equilibrium, but faster than in the absence of catalyst. In the presence of both  $\delta F(t)$ , and an enzyme with conformational transitions having a large  $\Delta\theta$  that allows the enzyme to absorb energy from the fluctuation, the reaction proceeds not to equilibrium, but to a non-equilibrium steady state given by eqn (1) (see Table 1). The ability of a simple enzyme to absorb energy from external driving and convert that energy into the formation and maintenance of a non-equilibrium steady state suggests that the ability to function as a molecular machine may be ubiquitous rather than requiring specialized characteristics.<sup>9–11</sup>

Recent work on a synthetic molecular pump<sup>12,13</sup> shows how, based on the principles of stochastic pumping, chemical systems can be designed and synthesized to carry out several functions normally associated with life.<sup>14</sup> These molecules recruit new material and self-assemble to make themselves more complex while dissipating input energy provided by an externally maintained oscillation of the redox potential. In principle, they can even be designed to self-replicate although this has not yet

been accomplished. Before discussing these fascinating experimental examples of a stochastic pump, let us first focus on the fundamental principles by which macromolecules harness energy to form and maintain non-equilibrium steady states.

## 2. Externally driving macromolecules

### a. Thermodynamic interaction between a molecule and its environment

Molecules interact with their environment through changes in intensive thermodynamic parameters (generalized forces),  $F$ , such as temperature, pressure, electric field strength, applied force, chemical potentials of ligands, *etc.*, that influence the relative free-energies of different states of the macromolecule according to the relation<sup>15</sup>

$$dG = \sum_i \theta_i dF_i - \mathcal{A} d\xi \quad (2)$$

where  $\mathcal{A}$  is the affinity and  $\xi$  is the extent of reaction. These two terms were introduced by de Donder in the 1920's. The extent of reaction is zero when there is only substrate, and unity when there is only product. In a non-fluctuating environment  $\mathcal{A} = 0$  when  $\frac{dG}{d\xi} = 0$ , and so the extent of reaction connects Gibb's free-energy with the concept of chemical affinity. The affinity and extent of reaction do not enter into our subsequent discussion.

The amplitude of the effect of a change in  $F_i$  depends on the canonically conjugate extensive thermodynamic parameters  $\theta_i$ . The change in internal energy due to the response of the system is given by  $dU = \sum_i F_i d\theta_i$ . Pairs of conjugate parameters are shown in Table 2.

The extensive parameters are properties of the macromolecule, while the intensive parameters are properties of the environment. The extensive parameters are defined in terms of the change in free-energy due to a change in the corresponding intensive parameter, with all other intensive parameters held constant, *i.e.*,  $\theta_i \equiv \left. \frac{\partial G}{\partial F_i} \right|_{F_j \neq i}$ .

The intensive parameters,  $F_i$ , can be used to "control" the molecular system from the outside. Rapid step (transient or

**Table 1** Illustration of the synergistic effect between a macromolecular catalyst (enzyme) and external fluctuations. Neither alone influences the equilibrium between S and P, but combined can lead to the formation of a non-equilibrium steady-state between substrate and product

$S \rightleftharpoons P$ [only]	$\frac{[P]}{[S]} \xrightarrow{\text{Slow}} K_{\text{eq}}$
$S \rightleftharpoons P$ [ $+\delta F(t)$ ]	$\frac{[P]}{[S]} \xrightarrow{\text{Slow}} K_{\text{eq}}$
$S \rightleftharpoons P$ [ $+E$ ]	$\frac{[P]}{[S]} \xrightarrow{\text{Fast}} K_{\text{eq}}$
$S \rightleftharpoons P$ [ $+E + \delta F(t)$ ]	$\frac{[P]}{[S]} \xrightarrow{\text{Fast}} K_{\text{eq}} \langle e^{\Delta\theta[\delta F(t') - \delta F(t'')]} \rangle$

**Table 2** Pairs of conjugate intensive and extensive thermodynamic parameters. The intensive parameters can be externally controlled, while the extensive parameters are properties of the macromolecule. The interaction between the generalized forces and generalized displacements is the cornerstone of thermodynamic control of molecular systems

Intensive parameter, generalized force $F_i$	Conjugate extensive parameter generalized displacement $\theta_i$
$P$ (pressure),	$V$ (molecular volume)
$T$ (temperature),	$S$ (molecular entropy)
$\vec{E}$ (electric field)	$\vec{p}$ (molecular dipole moment)
$\vec{H}$ (magnetic field)	$\vec{M}$ (molecular magnetization)
$\Delta\Psi$ (membrane potential)	$q$ (displacement charge)
$\vec{F}$ (applied force)	$\vec{l}$ (molecular extension)
$\mu_L$ (chemical potential of ligand)	$n_L$ (number of molecules of ligand bound)

jump methods) or oscillatory (stationary methods) changes in these parameters form the basis for relaxation kinetics by which very fast reactions and their mechanisms can be experimentally probed.<sup>16</sup> More recently in single molecule experiments an external pulling force,  $\vec{F}$ , exerted by, *e.g.*, an atomic force microscope, has been applied and the extension of the molecule,  $\vec{l}$ , monitored<sup>17</sup> or in the case of an enzyme it was proposed that the rate of catalysis itself could be followed.<sup>18</sup>

Time dependent changes of the  $F_i$ , whether arising from an electric field generator plugged into a wall socket,<sup>19</sup> spatial gradients of temperature, pH, and chemicals near a thermal vent,<sup>20</sup> or externally enforced time dependent changes of the pH or the redox potential,<sup>13</sup> provides a source of energy. The focus in the present Feature Article will be on how this energy is transduced to form and maintain a non-equilibrium steady state.

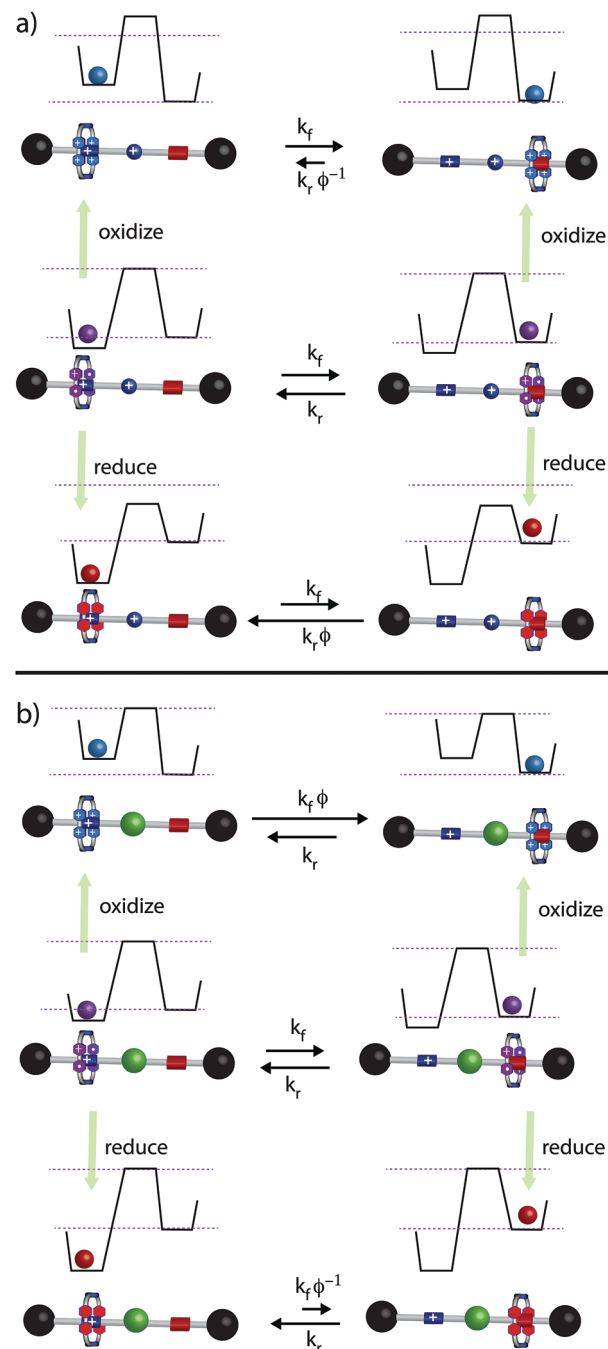
### b. Kinetics and thermodynamics of a molecular switch: the importance of kinetic asymmetry

We can solidify some of the basic concepts useful for understanding the effects of external fluctuations in the context of a molecular switch based on mechanically bonded molecules.<sup>21–23</sup> Consider the redox responsive switches shown in Fig. 1a and b comprising a long dumbbell rod (DB) with two distinct recognition sites for a cyclobisparaquat (CBPQT) ring through which the rod has been threaded. The rod is terminated on each end by a stopper group (often a di-isopropyl residue) that prevents the ring from slipping. The CBPQT ring DB complex forms a mechanically bonded molecule that can undergo switching triggered by changes in the redox potential of the solution. With a different design, similar mechanically bonded molecules can form the basis of true molecular machines that undergo directional motion and even perform mechanical or chemical work in the environment.

The CBPQT ring can exist in several different oxidation states,<sup>24</sup> ranging from a fully reduced (CBPQT<sup>0</sup>) neutral species (shown in red) to a partially oxidized diradical cation (CBPQT<sup>2(+•)</sup>) (shown in purple), to a fully oxidized (CBPQT<sup>4+</sup>) tetracation (shown in blue). The two recognition sites on the DB have different properties. One recognition site, shown in blue is charged, and so the interaction energy depends strongly on the charge on the CBPQT ring, and hence on its redox state. The other, shown in red, has an interaction energy that depends on its electron donating or withdrawing character and we take the interaction energy to be independent of the charge on the ring. Frasconi *et al.*<sup>24</sup> have provided a list of several possible recognition sites along with the interaction energies with CBPQT rings in different oxidation states.

The only difference between the structures in Fig. 1a and b is the moiety between the recognition sites that sets the energy barrier for switching. The design in Fig. 1a has a charged barrier (*e.g.*, a pyridinium group PY<sup>+</sup>) the effect of which is based on coulomb interaction, whereas the design in Fig. 1b has a “molecular speed bump” (*e.g.*, an isopropyl phenyl (IPP) group) where the barrier height depends only on steric interactions and is independent of the redox state of the CBPQT.

Because the interaction energy with the red recognition site is independent of the oxidation state of the CBPQT ring the free



**Fig. 1** Illustration of a mechanically bonded molecular switch triggered by redox potential. A dumb-bell shaped rod has two recognition sites for a CBPQT ring through which it is threaded. After threading, the rod is terminated by stopper groups that prevent the ring from escaping. One recognition site is charged and so its interaction with the ring is strongly modulated by the redox state of the ring. The interaction energy between the other recognition site and the ring is essentially independent of the redox state of the ring. The thermodynamic properties and state occupancies at equilibrium are identical for the systems in (a) and (b). There is however a difference in the kinetics. (a) The kinetic barrier that controls the rate of transition of the ring between the two recognition stations is provided by a charged residue (shown in blue), so the height of the barrier, measured relative to the reference level shown as a dotted line, depends on the oxidation state of the ring. (b) The kinetic barrier is provided by a molecular “speed bump” (shown in green) with a barrier height determined by steric interactions. The height of the barrier relative to the reference is independent of the oxidation state of the ring.

energies of all three states with the ring encircling the red recognition site are the same and can, without loss of generality, be set to zero. This is the reference energy shown by the dotted lines in Fig. 1a and b. The free energy of the state with the ring in the intermediate oxidation state, CBPQT<sup>2(+•)</sup> and encircling the blue recognition site is denoted  $-G_0$ . A coulombic interaction energy  $G_{\text{int}}$  must be subtracted from this amount to get the free-energy,  $-G_0 - G_{\text{int}}$ , of the system with the ring in the fully reduced state CBPQT<sup>0</sup> and the same amount is added to get the free-energy,  $-G_0 + G_{\text{int}}$ , of the system with the ring in the fully oxidized state CBPQT<sup>4+</sup>. The Coloumbic interaction energy,  $G_{\text{int}}$ , is about 15 kcal mol<sup>-1</sup>, somewhat greater than the energy released by ATP hydrolysis in biological systems. This energy is reflected in the term  $\phi = e^{G_{\text{int}}}$  that appears in the rate constants in Fig. 1. Note that each equilibrium constant, *i.e.*, the ratio of rate constants, in Fig. 1a is identical to the corresponding equilibrium constant in Fig. 1b, although in Fig. 1a, the dependence on  $G_{\text{int}}$  appears entirely in the backward rate constant because the energy of the transition state is modulated along with the energy of the complex of the ring with the blue recognition site. In contrast, in Fig. 1b the dependence on  $G_{\text{int}}$  appears entirely in the forward rate constants because the energy (relative to zero) of the transition state set by the neutral moiety due to steric interactions is independent of the charge on the ring. This difference is what is meant by the kinetic asymmetry of the system.

Now, consider the effect of very slowly varying the redox potential periodically between a value where all of the rings are fully oxidized to CBPQT<sup>4+</sup> and a value where all of the rings are fully reduced to the electrically neutral CBPQT<sup>0</sup> form, and where the lifetime under the strongly oxidizing condition is the same as the lifetime under the strongly reducing condition. Because of the slowness of the perturbation the system remains in equilibrium at all times, where the equilibrium is set by the instantaneous redox potential. The equilibrium concentrations of the different molecular species are independent of whether the system has an electrostatic or steric barrier between the two recognition sites – the response of the two different molecules shown in Fig. 1a and b are identical under slow driving by external changes of the redox potential. The effect of the oscillation always drives the time average ratio between [red] and [blue] towards unity.

If, however, the redox potential changes much faster than the relaxation time of the molecular system the situation is very different. In the limit that the fluctuations are very fast we can calculate the ratio of rings on the red and blue recognition sites by simply dividing the average of the forward rate constants by the average of the reverse rate constants. Since  $\phi \gg 1$ , the steady state ratio for the system with the electrostatic barrier is well approximated as

$$\frac{\sum [\text{red}]}{\sum [\text{blue}]}_{\text{ss}} \approx \frac{k_f}{k_r} \phi^{-1} \quad \text{electrostatic barrier}$$

whereas the corresponding ratio for the steric barrier is

$$\frac{\sum [\text{red}]}{\sum [\text{blue}]}_{\text{ss}} \approx \frac{k_f}{k_r} \phi \quad \text{steric barrier}$$

The identity of the moiety that sets the kinetic barrier and how the height of the barrier varies with the redox state of the ring is all important in determining whether non-equilibrium fluctuations of the redox potential favor binding to the blue recognition site or to the red recognition site at the non-equilibrium steady state driven by fast fluctuations of the redox potential.

### c. Time dependent forcing of a ligand binding reaction

The fluctuation of the redox potential acts on the molecular switch in Fig. 1 stoichiometrically.<sup>9</sup> Other generalized forces, such as temperature, pressure, electric field or membrane potential, act continuously. Consider a simple reaction in which a ligand, S, binds to a macromolecule, E, by the elementary mechanism  $S + E \rightleftharpoons E_L$ . An energy diagram for this reaction in the absence of external fluctuations is shown in Fig. 2, where  $\mu^0$  is a reference chemical potential of S, and  $\Delta G$  is the basic free energy difference between the free and bound form of the molecule E. The quantity  $\mu^0 - \Delta G$  plays the role of a “Fermi” level and can be either greater than, equal to, or less than zero. Fig. 2 shows the effect of an intensive parameter  $F(t) = F_0 + \delta F(t)$  that is externally caused to fluctuate with period  $T$  and amplitude  $\delta F_{\text{max}}$  where  $\delta F(t + T) = \delta F(t)$  and where  $\int_0^T \delta F(t) dt = 0$  on the energy levels of the enzyme for two cases, where we take state E as the reference. The effect of the fluctuation on the equilibrium constant, *i.e.*, on the energy level of state E<sub>L</sub>, is a thermodynamic identity,  $\frac{k_{+1}(t)}{k_{-1}(t)} = \frac{k_{+1}^0}{k_{-1}^0} \phi$ , with  $\phi \equiv e^{\delta F(t)\Delta\theta}$ . We take into account the possibility of kinetic asymmetry as discussed above by assigning the individual rate constants to be  $k_{+1}(t) = k_{+1}^0 \phi^{z_1}$  and  $k_{-1}(t) = k_{-1}^0 \phi^{(1-z_1)}$ . Often the forward rate is instead expressed as  $k_{+1}(t) = k_{+1}^0 e^{\Delta\theta^\ddagger \delta F(t)}$  where  $\Delta\theta^\ddagger$  is termed the generalized displacement of activation, with the reverse rate constant

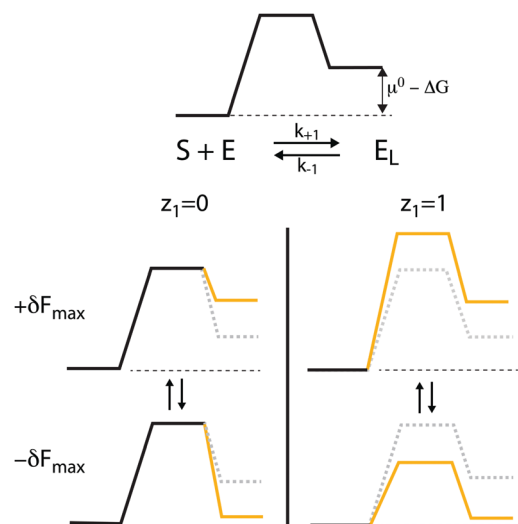


Fig. 2 Illustration of the effect of external fluctuation on the energy levels for a ligand binding reaction. Depending on  $z_1$  the external fluctuation either stabilized the bound state or the unbound state. The energy  $\mu^0 - \Delta G$  includes both the standard chemical potential of S and the basic free-energy difference of E and E<sub>L</sub>.



$k_{-1}(t) = k_{-1}^0 e^{(\Delta\theta^\ddagger - \Delta\theta)\delta F(t)}$ . The form we have chosen, with an apportionment constant ( $z_1$ ) is equivalent, and more convenient when considering cyclic processes such as catalysis, and the operation of molecular machines. The apportionment factor is a measure of whether  $\theta^\ddagger$  is closer to the value of the generalized displacement of the reactant, in which case  $\Delta\theta^\ddagger \approx 0 \Rightarrow z_1 \approx 0$ , or whether  $\theta^\ddagger$  is closer to the value of the generalised displacement of the product, in which case  $\Delta\theta^\ddagger \approx \Delta\theta \Rightarrow z_1 \approx 1$ . For example, if the volume of the transition state is closer to the volume of the reactant than to the product, pressure fluctuations will favor reactant, and *vice versa*.

For very slow fluctuations the system remains in equilibrium at every instant although the free energy that defines the equilibrium changes slowly in time. The average concentration  $\langle[E_L]\rangle$  can be calculated by taking the average of the equilibrium value over a cycle of the fluctuation,  $\frac{\langle[E_L]\rangle_{eq}}{E_{total}} = \frac{1}{T} \int_t^{t+T} \frac{[S]K_1^0 e^{\delta F(t)\Delta\theta}}{1 + [S]K_1^0 e^{\delta F(t)\Delta\theta}} dt$  where  $E_{total} = [E] + [E_L]$ . The straightforward, equilibrium effect of the fluctuation<sup>25,26</sup> leads always to a ratio  $\frac{\langle[E_L]\rangle_{eq}}{E_{total}}$  that is closer to 0.5 than when  $\delta F(t) = 0$ . In the limit  $\delta F_{max} \rightarrow \infty$ , the ratio  $\frac{\langle[E_L]\rangle_{eq}}{E_{total}} \rightarrow \frac{1}{2}$ , with the value oscillating between 1 and 0.

The effect of the external driving is much more interesting for faster fluctuations<sup>8,9</sup> where the individual rate constants come into play through correlations between the excess work  $\delta F(t)\Delta\theta$  and the rate as shown in Fig. 2 and in Table 3.

Having the rate of  $S + E \rightarrow E_L$  large when the excess work is positive (*i.e.*, is dissipative) for  $z_1 = 1$  allows the fluctuations to favor the bound form. Similarly, having the rate of  $E_L \rightarrow S + E$  large when the excess work is positive (*i.e.*, is dissipative) for  $z_1 = 0$  allows the fluctuations to favor the non-bound form.

We illustrate the importance of the kinetic asymmetry with a simple calculation. The instantaneous rate of change of  $[E_L]$  can be numerically evaluated from the first order ordinary differential equation with time dependent coefficients,

$$\frac{d[E_L]}{dt} = [S]k_{+1}^0 \phi^{-z_1}(E_{Tot} - [E_L]) - k_{-1}^0 \phi^{(1-z_1)}[E_L] \quad (3)$$

An example calculation for sinusoidal oscillation  $\delta F(t) = \delta F_{max} \cos(2\pi ft)$  is shown in Fig. 3, where we set  $[E_L](0) = 0.5E_T$ , and follow the non-driven relaxation ( $\delta F_{max} = 0$ , green curve), or the driven relaxation ( $\delta F_{max} = 1$ ) with  $z_1 = 1$  (orange curve) or with  $z_1 = 0$  (blue curve) toward the final steady state. The characteristic relaxation time  $\tau = ([S]k_{+1}^0 + k_{-1}^0)^{-1}$  is indicated on the graph, and is

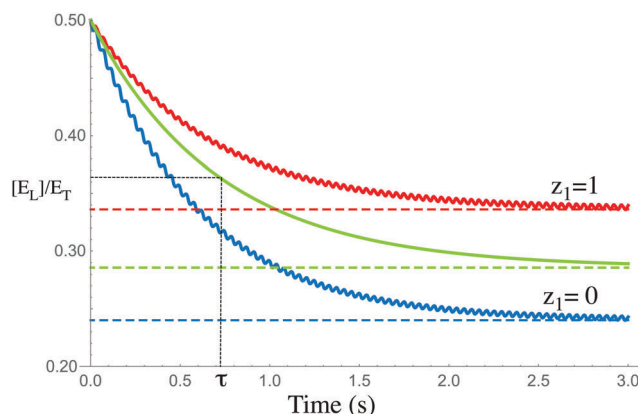


Fig. 3 Plots of  $\frac{[E_L]}{E_T}$  vs. time for  $\delta F(t) = \delta F_{max} \cos(2\pi ft)$  for  $\delta F_{max} = 0$  (green curve) and for  $\delta F_{max} = 1$  with  $z_1 = 1$  (orange curve) and  $z_1 = 0$  (blue curve). The inverse frequency  $f^{-1}$  is much smaller than the relaxation time of the binding process. The amplitude of the oscillations decreases with increasing  $f$ , but the final steady-state is independent of the frequency of the forcing.

defined as that time  $\tau$  at which  $|[E_L](\tau) - E_{L,ss}| = |E_L(0) - E_{L,ss}|/e$ . The frequency of driving is set to  $20\tau^{-1}$ . At larger frequency, the oscillation amplitude is very small and imperceptible, but, for  $f \gg \tau^{-1}$ , the driving induced steady state ratio is independent of  $f$ .

As expected based on physical arguments, the kinetic apportionment factor controls whether the external driving favors or disfavors the bound state  $E_L$ . When  $z_1 = 1$ , the transition  $S + E \rightarrow E_L$  occurs mainly when  $\delta F < 0$  since the barrier relative to the energy of state  $E$  is low. The reverse transition  $E_L \rightarrow E + S$  does not depend on  $\delta F$  since the barrier relative to the energy of state  $E_L$  does not change. In contrast, when  $z_1 = 0$ , the transition  $E_L \rightarrow E + S$  occurs mainly when  $\delta F > 0$  since the barrier relative to the energy of state  $E_L$  is low, and the reverse transition  $S + E \rightarrow E_L$  is independent of  $\delta F$  since the barrier relative to the energy of state  $E$  does not change.

For  $T \ll \tau$ , there will be either zero or one transition per molecule during each period. In this case, the kinetics can be described in terms of the time averages of the rate constants,  $\frac{d[E_L]}{dt} = [E][S]\overline{k_{+1}} - [E_L]\overline{k_{-1}}$  and the ratio of the probability for binding vs. dissociation over a period is

$$\frac{\pi(E \rightarrow E_L)}{\pi(E_L \rightarrow E)} = \frac{[E][S] \int_t^{t+T} k_{+1}(t) dt}{[E_L] \int_t^{t+T} k_{-1}(t) dt} \quad (4)$$

**Table 3** Summary of the thermodynamic and kinetic correlations for a ligand binding reaction in a fluctuating environment. The bold text indicates those correlations by which the bound form is favoured when  $z_1 = 1$ , and the unbound form is favoured when  $z_1 = 0$

	$-\delta F_{max}$		$+\delta F_{max}$	
$S + E \rightarrow E_L$	$\mathcal{F}, \Delta\theta\delta F_{max}$		$\mathcal{B}_R, -\Delta\theta\delta F_{max}$	
	$z_1 = 0$ no change	$z_1 = 1$ rate +	$z_1 = 0$ no change	$z_1 = 1$ rate –
$E_L \rightarrow S + E$	$\mathcal{F}_R, -\Delta\theta\delta F_{max}$		$\mathcal{B}, \Delta\theta\delta F_{max}$	
	$z_1 = 0$ rate –	$z_1 = 1$ no change	$z_1 = 0$ rate +	$z_1 = 1$ no change

where  $\int_t^{t+T} k_{+1}(t) dt \equiv \overline{k_{+1}}$  and  $\int_t^{t+T} k_{-1}(t) dt \equiv \overline{k_{-1}}$ . The “steady-state” occurs when  $\pi(E \rightarrow E_L) = \pi(E_L \rightarrow E)$ . By use of the identity  $[S]k_{+1}(t) = k_{-1}(t)K_1^0 e^{\mu_S - \mu^0} e^{-\Delta\theta\delta F(t)}$  in the numerator of eqn (4) we derive

$$\left. \frac{[E_L]}{[E]} \right|_{ss} = K_1^0 e^{\mu_S - \mu^0} \underbrace{\frac{\int_t^{t+T} k_{-1}(t) e^{-\Delta\theta\delta F(t')} dt}{\int_t^{t+T} k_{-1}(t) dt}}_{\langle e^{-\Delta\theta\delta F(t)} \rangle} \quad (5)$$

The eqn (5) is the exponential of the work done in the transition  $E_L \rightarrow S + E$  averaged over the ensemble of times at which this transition can occur and weighted by the probability (rate constant) of the reverse transition at each time. For sinusoidal oscillation  $\delta F(t) = \delta F_{\max} \cos(2\pi ft)$  we find that for  $z_1 = 0$ ,  $\langle e^{-\Delta\theta\delta F(t)} \rangle = I_0(\delta F_{\max} \Delta\theta)$  and for  $z_1 = 1$ ,  $\langle e^{-\Delta\theta\delta F(t)} \rangle = I_0^{-1}(\delta F_{\max} \Delta\theta)$  where  $I_0(x)$  is the zeroth order modified Bessel function of the first kind. External fluctuations,  $\delta F(t)$ , favor the state that the transition state most resembles with respect to the conjugate parameter  $\theta$ . The position of the non-equilibrium steady-state is determined kinetically by the apportionment factor  $z_1$ . In contrast to equilibrium where the thermodynamic properties of the reactant and product specify the concentration ratio, the properties of the transition state are also important for determining the non-equilibrium steady state ratio.

#### d. General result for external work induced non-equilibrium steady-states

The theory of stochastic pumping is grounded by the principle of microscopic reversibility<sup>27–31</sup> (MR) according to which, irrespective of whether the system is in thermodynamic equilibrium or not, the ratio of conditional probabilities for a forward and microscopic reverse path between any two states  $i$  and  $j$ , of some molecule of interest is given as the exponent of the basic free-energy difference  $\Delta G_{ij} = G_i - G_j$  between the states plus the excess work exchanged with the environment  $\mathcal{W}_S = \int (dG_i - dG_j)$  in the forward path<sup>27</sup>  $S$  from  $i$  to  $j$

$$\frac{\pi(i \xrightarrow{S} j)}{\pi(j \xrightarrow{S^\dagger} i)} = e^{\Delta G_{ij} + \mathcal{W}_S} \quad (6)$$

The free energy change evaluated at some standard condition,  $\Delta G_{ij} = G_i - G_j$ , is a state function – i.e., it is the same for every path  $S$  irrespective of whether the system is in equilibrium or not. The excess work  $\mathcal{W}_S$  however depends on the path  $S$ . We take the sign convention that  $\mathcal{W}_S$  is positive when energy is absorbed from the source of the fluctuation and dissipated in the environment in the transition  $i \xrightarrow{S} j$ . The microscopic reverse of  $S$  is denoted  $S^\dagger$ , and the symmetry relation  $\mathcal{W}_{S^\dagger} = -\mathcal{W}_S$  for all  $S$ .

For a process described as a Markov chain eqn (6) follows from the definition of the equilibrium constants between the states in the chain. For a continuous process eqn (6) is easily derived<sup>27</sup> by using a theory presented by Onsager and Machlup in 1953.<sup>32</sup> The Onsager/Machlup approach is notable for its emphasis on trajectories rather than on states, and is especially

important in the recognition that the excess work,  $\mathcal{W}_S$ , due to changes of the intensive thermodynamic parameters away from their reference values is a property of a trajectory and not of the end-states.

The steady-state ratio between concentrations  $[i]$  and  $[j]$  is obtained by summing the probabilities for all paths from  $j$  to  $i$  multiplied by  $[j]$  and dividing by the sum of the probabilities for all paths from  $i$  to  $j$  multiplied by  $[i]$  and setting that ratio to unity. We then use eqn (6) to derive an extension of the Boltzmann expression for the ratio of probabilities to be in two states at a not necessarily equilibrium steady state

$$\left. \frac{[i]}{[j]} \right|_{ss} = \frac{\sum_n \pi_{S_n} \left( j \xrightarrow{S_n} i \right)}{\sum_n \pi_{S_n} \left( i \xrightarrow{S_n} j \right)} = e^{-\Delta G_{ij}} \frac{\sum_n \pi_{S_n} \left( i \xrightarrow{S_n} j \right) e^{-\mathcal{W}_{S_n}}}{\underbrace{\sum_n \pi_{S_n} \left( i \xrightarrow{S_n} j \right)}_{\langle e^{-\mathcal{W}_{S_n}} \rangle}} \quad (7)$$

The second equality arises by application of the identity, eqn (6), to each term in the numerator of eqn (7). The term  $e^{-\Delta G_{ij}}$  is the standard Boltzmann factor familiar from equilibrium statistical mechanics. The apparent simplicity of the second factor,  $\langle e^{-\mathcal{W}_{S_n}} \rangle$ , belies the fact that away from equilibrium the ensemble average of the exponential of the excess work over all paths is non-trivial to calculate, and can be either less than, equal to, or greater than unity. As we saw with the specific concrete example of ligand binding in the presence of fluctuations, the kinetic apportionment factor is the key quantity determining whether the fluctuations favor, relative to the equilibrium, state  $i$  or state  $j$  in the non-equilibrium steady state.

The expression  $\left. \frac{[i]}{[j]} \right|_{ss} = e^{-\Delta G_{ij}} \langle e^{-\mathcal{W}_{S_n}} \rangle$  is very general and applies not only to the effects of external fluctuations but also to non-equilibrium steady states driven by chemical fuel, to pH fluctuations, and to thermal cycling between cool and hot regions. The key necessity is to correctly calculate the ensemble average of the exponent of the excess work  $\mathcal{W}_S$ , an endeavor that is greatly facilitated by recognition that the paths come in symmetry related quartets.

#### e. Fundamental symmetry relations for stochastic pumps<sup>33,34</sup>

Consider the four paths, two,  $\mathcal{F}$  and  $\mathcal{B}_R$ , from  $i$  to  $j$ , and two,  $\mathcal{B}$  and  $\mathcal{F}_R$  from  $j$  to  $i$ . These four paths are distinguished from one another by the change in the sum of the free energy and the excess work exchanged with the environment.<sup>33–36</sup> The sign of the free energy change,  $\Delta G_{\mathcal{F}} = \Delta G_{\mathcal{B}_R} = \Delta G_{ij}$  and  $\Delta G_{\mathcal{B}} = \Delta G_{\mathcal{F}_R} = -\Delta G_{ij}$  depends on the direction, whereas the work exchanged with the environment depends on whether the fluctuating parameter has the value  $+\delta F$  or  $-\delta F$  at the moment the transition occurs. For the ligand binding process these paths are summarized in Table 3. We find the ratio of the sum of the path probabilities leading from  $j$  to  $i$  to the sum of the path probabilities leading from  $i$  to  $j$

$$\frac{\pi_{\mathcal{F}_R} + \pi_{\mathcal{B}}}{\pi_{\mathcal{F}} + \pi_{\mathcal{B}_R}} \approx e^{-\Delta G_{ij}} \left( \frac{\pi_{\mathcal{F}} e^{-\mathcal{W}} + \pi_{\mathcal{B}_R} e^{+\mathcal{W}}}{\pi_{\mathcal{F}} + \pi_{\mathcal{B}_R}} \right) \quad (8)$$

**Table 4** Relations based on microscopic reversibility between the four symmetry related paths for any transition in the presence of fluctuations

Path	Net work	Path probabilities
$\mathcal{F} = i \xrightarrow{\mathcal{W}_{\mathcal{F}}} j$	$-\Delta G_{ij} + \mathcal{W}$	$\pi_{\mathcal{F}}$
$\mathcal{F}_{\text{R}} = j \xrightarrow{\mathcal{W}_{\mathcal{F}_{\text{R}}}} i$	$\Delta G_{ij} - \mathcal{W}$	$\pi_{\mathcal{F}_{\text{R}}} = \pi_{\mathcal{F}} e^{+\Delta G_{ij} - \mathcal{W}}$
$\mathcal{B}_{\text{R}} = i \xrightarrow{\mathcal{W}_{\mathcal{B}_{\text{R}}}} j$	$-\Delta G_{ij} - \mathcal{W}$	$\pi_{\mathcal{B}_{\text{R}}}$
$\mathcal{B} = j \xrightarrow{\mathcal{W}_{\mathcal{B}}} i$	$\Delta G_{ij} + \mathcal{W}$	$\pi_{\mathcal{B}} = \pi_{\mathcal{B}_{\text{R}}} e^{-\Delta G_{ij} + \mathcal{W}}$

If  $\pi_{\mathcal{F}} = \pi_{\mathcal{B}}$  the term in parenthesis is unity. In this case, the steady state ratio between states  $i$  and  $j$ , despite significant continual dissipation due to the fluctuation, is the same as at equilibrium,  $e^{-\Delta G_{ij}}$ . For  $\mathcal{W} > 0$ , if  $\pi_{\mathcal{F}} > \pi_{\mathcal{B}}$  the term in parenthesis is less than unity, and if  $\pi_{\mathcal{F}} < \pi_{\mathcal{B}}$  the term in parenthesis is greater than unity.

The symmetry relationships in Table 4 hold not only for external fluctuation driven non-equilibrium steady-states, but also for driving the formation of a non-equilibrium steady state or performance of work by chemical catalysis such as ATP hydrolysis (H) coupled to some chemical, mechanical, or osmotic step (S).<sup>35,36</sup> There are often only the four paths described  $\mathcal{F} = (+\text{H}, +\text{S})$ ,  $\mathcal{B} = (+\text{H}, -\text{S})$ , and their microscopic reverses  $\mathcal{F}_{\text{R}} = (-\text{H}, -\text{S})$ , and  $\mathcal{B}_{\text{R}} = (-\text{H}, +\text{S})$ , with  $-\text{H}$  denoting ATP synthesis.

At equilibrium,  $\mathcal{W} = 0$ , in which case  $[i]\pi_{\mathcal{F}} = [j]\pi_{\mathcal{F}_{\text{R}}}$  and  $[i]\pi_{\mathcal{B}_{\text{R}}} = [j]\pi_{\mathcal{B}}$ . This is a condition known as detailed balance. Detailed balance is a corollary of microscopic reversibility and holds only at equilibrium. Most biological systems operate far from equilibrium ( $|\mathcal{W}| \gg 1 \Rightarrow \pi_{\mathcal{F}} \gg \pi_{\mathcal{F}_{\text{R}}}, \pi_{\mathcal{B}} \gg \pi_{\mathcal{B}_{\text{R}}}$ ). The condition  $[i](\pi_{\mathcal{F}} + \pi_{\mathcal{B}_{\text{R}}}) = [j](\pi_{\mathcal{B}} + \pi_{\mathcal{F}_{\text{R}}})$  indicates steady state between  $i$  and  $j$  but does not guarantee that the system is in equilibrium or that “detailed balance” holds.

Many biological motors are tightly coupled such that every chemical, mechanical, or osmotic step is accompanied by an ATP hydrolysis, and *vice versa* ( $\pi_{\mathcal{F}} \gg \pi_{\mathcal{B}}$ ). These conditions can form the basis for simplifying approximations. In the limit that

$\frac{\pi_{\mathcal{B}}}{\pi_{\mathcal{F}}} \rightarrow 0$  the steady state ratio from eqn (8) is  $\left[\frac{j}{i}\right]_{\text{ss}} \approx e^{-\Delta G_{ij} - \mathcal{W}}$ .

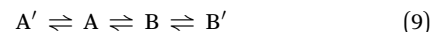
This is known as the thermodynamic control regime. In the limit

$\mathcal{W} \rightarrow \infty$ , we have  $\left[\frac{j}{i}\right]_{\text{ss}} \approx \frac{\pi_{\mathcal{B}}}{\pi_{\mathcal{F}}}$  known as the kinetic control regime.<sup>36</sup>

## f. Selection principle for fluctuation induced adaptation

The theory of stochastic pumping<sup>8,9</sup> shows that external fluctuations select trajectories according to both dissipation and kinetic asymmetry. External fluctuations always lead to increased dissipation but in the absence of kinetic asymmetry the dissipation does not lead to symmetry breaking by which a non-equilibrium steady state is formed or by which chemical, osmotic, electrical, or mechanical work is done. In the ensemble average the decisive factor in determining the position of the non-equilibrium steady-state or the direction of work is the kinetic asymmetry of the system since for every forward trajectory there is an equally dissipative backward trajectory.

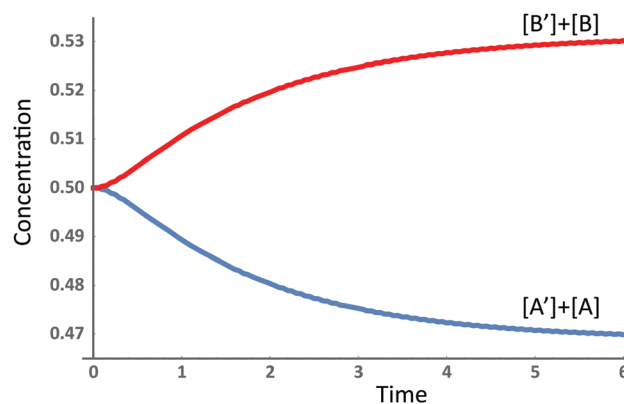
A very recent theory known as “dissipative adaptation”<sup>37,38</sup> proposes, in contrast, that a fluctuation induced steady-state adapts to dissipate maximum energy in the environment, with no mention of kinetic asymmetry. We can easily distinguish the recent “dissipative adaptation” model from the earlier stochastic pump theory by considering transitions between two macroscopic states  $A = (A, A')$  and  $B = (B, B')$



where for simplicity we take  $G_{A'}^0 = G_A^0 = G_B^0 = G_{B'}^0 = G^0$  so that at equilibrium and with  $F = 0$  all four microscopic states will have equal concentrations. We further take all unperturbed rate constants to be unity and then choose the extensive thermodynamic parameters  $\theta_A^0 = \theta_B^0 = \theta_{A'}^0 = \theta_{B'}^0 = \theta^0$ ,  $\theta_{A'}^0 = 3\theta^0$ ,  $\theta_{B'}^0 = 2\theta^0$  so that in the presence of fluctuations  $\delta F(t)$  the transitions  $A' \rightleftharpoons A$ , with  $\Delta\theta_{A,A'} = 2\theta^0$ , will dissipate more energy than the transitions  $B \rightleftharpoons B'$  with  $\Delta\theta_{B,B'} = \theta^0$ . The differential equations describing this model are

$$\begin{aligned} \frac{d[A']}{dt} &= \phi^{2z_A} [A] - \phi^{2(z_A-1)} [A'] \\ \frac{d[A]}{dt} &= \phi^{2(z_A-1)} [A'] + [B] - (1 + \phi^{2z_A}) [A] \\ \frac{d[B]}{dt} &= [A] + \phi^{z_B-1} [B'] - (1 + \phi^{z_B}) [B] \end{aligned} \quad (10)$$

where  $[B'] = ([\text{total}] - [A'] - [A] - [B])$  and where  $\phi = e^{\delta F_{\text{max}} \theta^0 \cos(2\pi f t)}$ . The time evolution of  $[A] + [A']$  and of  $[B] + [B']$  obtained by numerical computation of eqn (9) is shown in Fig. 4, where we set the kinetic parameters to be  $z_A = 1/2$  and  $z_B = 1$  to break the kinetic symmetry in macro-state B but not in A. According to the dissipative adaptation model,  $[A] = [A] + [A']$  should, at steady state, be larger than  $[B] = [B] + [B']$  because of the enhanced dissipation in state A. In contrast, the earlier stochastic pump model for adaptation predicts that  $[B]$  should be larger than  $[A]$  because of the broken kinetic symmetry of the state B (*i.e.*,  $z_B = 1$ ) in combination with the non-zero but modest dissipation in the



**Fig. 4** Illustration of the effect of fluctuations,  $\delta F(t)$ , on the reaction eqn (8), where states A are more effective at dissipating energy, but the kinetic symmetry of states B, but not A is broken. Maximum dissipation would occur with  $[B] = [B'] = 0$ , but instead, because of the broken symmetry, states B are favoured by the fluctuation.

transition  $B \rightleftharpoons B'$ . The results are unequivocal – the behaviour of the system is consistent with the prediction of the stochastic pump model for adaptation<sup>8</sup> and is not consistent with the prediction of dissipative adaptation.<sup>37,38</sup> The kinetic apportionment constants play an ineluctably important role in determining the position of the fluctuation driven non-equilibrium steady state. With the parameters used in the calculations the transitions  $A \rightarrow A'$  and the transitions  $A' \rightarrow A$  both dissipate, on average, a lot of, and equal amounts of energy. In contrast, the transitions  $B \rightarrow B'$  dissipate, on average, a moderate amount of energy, while the transitions  $B' \rightarrow B$  on average dissipate no energy. When we set  $\theta_{B'}^0 = \theta^0$  the external fluctuation does not interact at all with the transitions  $B \rightleftharpoons B'$ , but even in this case there is no tendency to favor state A if  $z_A = 1/2$ .

The basic principle is that fluctuations tend to select states where transitions into the state dissipate, on average, more energy than transitions out of the state, and that the magnitude of the effect depends on the difference in the average energy dissipated in the two directions.

The kinetic model eqn (9) of a very dissipative but kinetically symmetric macroscopic state A, connected to a less dissipative but kinetically asymmetric macroscopic state B is a single counter-example to the claim of the “dissipative adaptation” model that molecular systems adapt so as to dissipate a maximum amount of energy. However, I am aware of no evidence whatsoever in any system that dissipation without kinetic asymmetry can be harnessed for molecular adaptation or for any useful molecular function other than heating. Molecular systems simply do not adjust to dissipate the most energy. Instead, the effect of fluctuations is kinetically determined as predicted by the stochastic pumping model.<sup>8,9</sup>

The kinetic asymmetry emphasized by the stochastic pumping model can be used by evolution to great effect in designing enzymes – macromolecular catalysts – to harvest energy from external fluctuation to do chemical, mechanical, and/or electrical work on their environment. While it is certainly true that those transitions in which a large amount of energy is dissipated are thermodynamically selected out of the ensemble of trajectories available to a system (see Table 4, where  $\pi_{\mathcal{F}}$  and  $\pi_{\mathcal{B}}$  are favored by the factor  $e^{+W}$  relative to their microscopic reverses), the essential point, as clarified in the work on stochastic pumping<sup>8,9</sup> and on Brownian motors,<sup>33,34</sup> is the kinetic symmetry breaking by which dissipative trajectories in one direction are kinetically blocked ( $\pi_{\mathcal{F}} \gg \pi_{\mathcal{B}}$ ), leaving behind those dissipative trajectories in the other direction. This kinetic asymmetry can lead to adaptation, and to the possibility for performance of work in the environment.

### 3. Stochastically pumping enzymes<sup>8</sup>

Consider an enzyme that catalyzes a reaction  $S \rightleftharpoons P$  by binding/releasing substrate, S, and product, P, in separate processes according to the Michaelis–Menten mechanism shown at the bottom of Fig. 5. With externally applied periodic fluctuations  $\delta F(t)$  a specific trajectory in which substrate is converted to

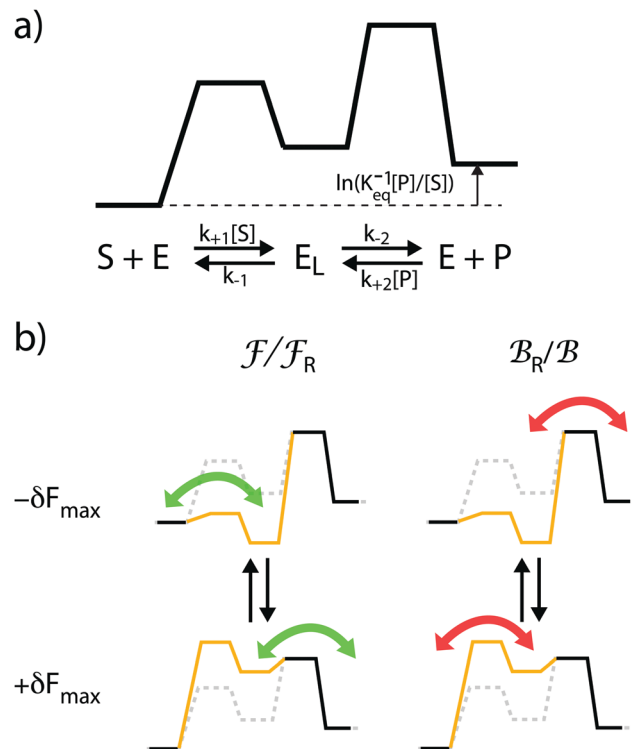


Fig. 5 (a) Illustration of a Michaelis–Menten enzyme where  $\Delta\mu = \ln\left(K_{eq}\frac{[S]}{[P]}\right)$  is the difference in chemical potential of substrate and product. (b) The effect of environmental fluctuations on the energy levels for a Michaelis–Menten enzyme the most probable trajectory involves binding S when the fluctuation has the value  $-\delta F_{max}$  and dissociation of P when the fluctuation has the value  $+\delta F_{max}$ . In this way the enzyme is able to use the excess dissipated energy to drive thermodynamically uphill conversion of S to P to form a non-equilibrium steady state as given in eqn (1). There is also a backward path in which P binds when the fluctuation has the value  $-\delta F_{max}$  and S is released when the fluctuation has the value  $+\delta F_{max}$ .

product involves binding S at time  $nT + t'$  by absorbing/dissipating energy  $\Delta G - \mu^0 + \mu_S + \Delta\theta\delta F(t')$  from the environment and releasing P at time  $nT + t''$  while dissipating/absorbing energy  $\Delta G - \mu^0 + \mu_P + \Delta\theta\delta F(t'')$  into the environment. The total energy absorbed/dissipated in the process is  $\Delta\mu + [\Delta\theta\delta F(t') - \Delta\theta\delta F(t'')]$ . The microscopic reverse of that process involves binding P at time  $nT + t''$  and releasing S at time  $(n + 1)T + t'$ .

We can calculate the ratio of the net probabilities for  $S \rightarrow P$  vs.  $P \rightarrow S$  by integrating over a period of the fluctuation

$$\frac{\pi(S \rightarrow P)}{\pi(P \rightarrow S)} = K_{eq} \frac{[S]}{[P]} \underbrace{\frac{\int \int_{t'}^{t'+T} k_{-1}(t') k_{+2}(t'') e^{-\Delta\theta[\delta F(t') - \delta F(t'')]} dt' dt''}{\int \int_{t'}^{t'+T} k_{-1}(t') k_{+2}(t'') dt' dt''}}_{\langle e^{-\Delta\theta\Delta\delta F(t)} \rangle} \quad (11)$$

We used the relations  $[S]k_{+1}(t') = k_{-1}(t')K_1^0 e^{\mu_S - \mu^0} e^{-\Delta\theta\delta F(t')}$  and  $[P]k_{+2}(t'') = k_{-2}(t'')K_2^0 e^{\mu_P - \mu^0} e^{-\Delta\theta\delta F(t'')}$  and the identity  $\frac{K_1^0 e^{\mu_S - \mu^0}}{K_2^0 e^{\mu_P - \mu^0}} = K_{eq} \frac{[S]}{[P]}$  to arrive at eqn (11). While presented as a time integral eqn (11) is better understood as an ensemble average over all



possible values of  $\delta F(t)$  for the binding and dissociation reactions. Trajectories involving binding at  $t''$  and dissociation at  $t'$  do not violate causality for periodic systems since  $t' + T > t''$ .

We calculate the non-equilibrium steady state by setting the ratio of probabilities in eqn (11) to equal unity,

$$\frac{[P]}{[S]}_{ss} = K_{eq} \left\langle e^{-\Delta\theta\Delta\delta F(t',t'')} \right\rangle \quad (12)$$

For the case that  $F(t) = \delta F_{max} \cos(2\pi ft)$  and that  $z_1 = 1, z_2 = 0$  as shown in Fig. 5 we evaluate the integral to find<sup>25</sup>  $\frac{[P]}{[S]}_{ss} = K_{eq} I_0^2(\delta F_{max} \Delta\theta)$ .

In the example<sup>8</sup> the ratio  $\frac{k_{+1}(t)k_{-2}(t)}{k_{+2}(t)k_{-1}(t)} = K_{eq}$  is time independent at every instant. Nevertheless, the condition  $\frac{k_{+1}(t)k_{-2}(t)[S]}{k_{+2}(t)k_{-1}(t)[P]} = 1$  clearly does not guarantee that the system is in equilibrium. Despite this fact, the claim that  $\frac{k_{+1}k_{-2}[S]}{k_{+2}k_{-1}[P]} = 1$  is a necessary and sufficient condition for the system to be in equilibrium, or that the entropy production is zero if and only if this condition holds, is still commonly published by well-respected authors in prominent books on molecular motors, feature articles, and major reviews in physics and physical chemistry journals, as well as in the primary literature.

While disagreements about interpretation of results, or about the best perspective from which to view a particular system can be important for scientific progress, the publication of mathematical assertions that are deductively false benefits no one.

The process shown in Fig. 5 can be mapped onto a single kinetic diagram if we consider a dichotomic fluctuation that can be described as a rate process  $+\delta F_{max} \xrightleftharpoons[\gamma]{\gamma} \delta F_{max}$ . The overall reaction can be described in terms of kinetic cycle diagrams and analyzed according to Terrell Hill's approach<sup>39</sup> as described in appendix c of ref. 9. We can also gain insight by considering the eight possible paths shown in Table 5. The terms in the numerator of the ensemble averages (e.g., eqn (7)) are products of a kinetic weighting factor and a thermodynamic term, both of which are given explicitly in Table 5. The condition for steady state is

$$\pi_{\mathcal{F}} + \pi_{\mathcal{B}_R} + \pi_{\mathcal{C}_I} + \pi_{\mathcal{C}_{II}} = \pi_{\mathcal{F}_R} + \pi_{\mathcal{B}} + \pi_{\mathcal{C}_{I,R}} + \pi_{\mathcal{C}_{II,R}}$$

which is equivalent to the relation

$$\frac{\pi_{\mathcal{F}} e^{-\mathcal{W}_{\mathcal{F}}} + \pi_{\mathcal{B}_R} e^{-\mathcal{W}_{\mathcal{B}_R}} + \pi_{\mathcal{C}_I} e^{-\mathcal{W}_{\mathcal{C}_I}} + \pi_{\mathcal{C}_{II}} e^{-\mathcal{W}_{\mathcal{C}_{II}}}}{\pi_{\mathcal{F}} + \pi_{\mathcal{B}_R} + \pi_{\mathcal{C}_I} + \pi_{\mathcal{C}_{II}}} \Big|_{ss} = 1 \quad (13)$$

Similar symmetry relations to those given in Table 5 apply even in complicated systems with more than two states and with several possible values for the fluctuating quantity. By induction we write

$$\frac{\sum_S \pi_S e^{-\mathcal{W}_S}}{\sum_S \pi_S} \Big|_{ss} = 1 \Leftrightarrow \frac{\sum_S \pi_S e^{-\mathcal{W}_{S,ex}}}{\sum_S \pi_S} \Big|_{ss} = e^{\Delta\mu_{ss}} \quad (14)$$

The sum is over all paths  $\mathcal{S}$  from  $S$  to  $P$ , where  $\mathcal{W}_S$  is the total work exchanged with the environment in path  $\mathcal{S}$ , and  $\mathcal{W}_{S,ex} (= \pm 2\Delta\theta\delta F_{max})$  for the dichotomic fluctuations used to generate Table 5) is the “excess” work due to the fluctuations, and where  $\Delta\mu_{ss} = -\ln\left(K_{eq} \frac{[S]}{[P]} \Big|_{ss}\right)$  is the difference in chemical potential between substrate and product at steady state. Using eqn (14) the steady state chemical potential difference can be calculated in terms of the “excess” work  $\mathcal{W}_{S,ex}$  exchanged between the enzyme and its fluctuating environment in each path. It is very important to avoid misidentifying  $\mathcal{W}_{S,ex}$  as the “dissipated work”, since dissipation is positive definite, and the sign of the excess work can be either positive or negative depending on the kinetic details of the system.

Let us step back and see how this result sheds light on the fundamental mechanisms by which a macromolecule can harness energy from environmental fluctuations to do work. Consider a chemical system in which the reaction  $S \rightleftharpoons P$  is at equilibrium. We then turn on some fluctuation  $\delta F_i(t)$ . If the conjugate extensive parameters for  $S$  and  $P$  are the same,  $\theta_{i,S} = \theta_{i,P}$ , the reaction will not absorb energy from the fluctuation and will remain in equilibrium. We now add a tiny amount of an enzyme that catalyzes the reaction  $S \rightleftharpoons P$  where the catalytic mechanism involves conformational changes for which the  $\Delta\theta \neq 0$ . The enzyme will therefore absorb energy from the fluctuation and drive the reaction away from equilibrium. The kinetic asymmetry of the interaction between the barriers and the environmental fluctuation determine whether  $S$  or  $P$  is favored relative to equilibrium. These results suggest that

**Table 5** Summary of the thermodynamics and kinetics of possible paths for interconversion between substrate ( $S$ ) and product ( $P$ ) by an enzyme in a dichotomously fluctuating environment

Path	Thermodynamic term	Kinetic weight
$\mathcal{F} = S + E \xrightarrow{-\delta F_{max}} E_L \xrightarrow{+\delta F_{max}} E + P$	$e^{\mathcal{W}_{\mathcal{F}}} = e^{2\Delta\theta\delta F_{max}} e^{-\Delta\mu}$	$\pi_{\mathcal{F}} = [S]k_{+1}^0 k_{-2}^0 \gamma^2 e^{z_1 \Delta\theta\delta F_{max}} e^{(1-z_2)\Delta\theta\delta F_{max}}$
$\mathcal{F}_R = P + E \xrightarrow{+\delta F_{max}} E_L \xrightarrow{-\delta F_{max}} E + S$	$e^{\mathcal{W}_{\mathcal{F}_R}} = e^{-\mathcal{W}_{\mathcal{F}}} = e^{-2\Delta\theta\delta F_{max}} e^{\Delta\mu}$	$\pi_{\mathcal{F}_R} = [P]k_{+2}^0 k_{-1}^0 \gamma^2 e^{(1-z_1)\Delta\theta\delta F_{max}} e^{z_2 \Delta\theta\delta F_{max}} = \pi_{\mathcal{F}} e^{-\mathcal{W}_{\mathcal{F}}}$
$\mathcal{B}_R = S + E \xrightarrow{+\delta F_{max}} E_L \xrightarrow{-\delta F_{max}} E + P$	$e^{\mathcal{W}_{\mathcal{B}_R}} = e^{-2\Delta\theta\delta F_{max}} e^{-\Delta\mu}$	$\pi_{\mathcal{B}_R} = [S]k_{+1}^0 k_{-2}^0 \gamma^2 e^{-z_1 \Delta\theta\delta F_{max}} e^{-(1-z_2)\Delta\theta\delta F_{max}}$
$\mathcal{B} = P + E \xrightarrow{-\delta F_{max}} E_L \xrightarrow{+\delta F_{max}} E + S$	$e^{\mathcal{W}_{\mathcal{B}}} = e^{-\mathcal{W}_{\mathcal{B}_R}} = e^{2\Delta\theta\delta F_{max}} e^{\Delta\mu}$	$\pi_{\mathcal{B}} = [P]k_{+2}^0 k_{-1}^0 \gamma^2 e^{-(1-z_1)\Delta\theta\delta F_{max}} e^{-z_2 \Delta\theta\delta F_{max}} = \pi_{\mathcal{B}_R} e^{-\mathcal{W}_{\mathcal{B}_R}}$
$\mathcal{C}_I = S + E \xrightarrow{+\delta F_{max}} E_L \xrightarrow{+\delta F_{max}} E + P$	$e^{\mathcal{W}_{\mathcal{C}_I}} = e^{-\Delta\mu}$	$\pi_{\mathcal{C}_I} = [S]k_{+1}^0 k_{-2}^0 e^{-z_1 \Delta\theta\delta F_{max}} e^{(1-z_2)\Delta\theta\delta F_{max}}$
$\mathcal{C}_{I,R} = P + E \xrightarrow{+\delta F_{max}} E_L \xrightarrow{+\delta F_{max}} E + S$	$e^{\mathcal{W}_{\mathcal{C}_{I,R}}} = e^{-\mathcal{W}_{\mathcal{C}_I}} = e^{\Delta\mu}$	$\pi_{\mathcal{C}_{I,R}} = [P]k_{+2}^0 k_{-1}^0 e^{(1-z_1)\Delta\theta\delta F_{max}} e^{-z_2 \Delta\theta\delta F_{max}} = \pi_{\mathcal{C}_I} e^{-\mathcal{W}_{\mathcal{C}_I}}$
$\mathcal{C}_{II} = S + E \xrightarrow{-\delta F_{max}} E_L \xrightarrow{-\delta F_{max}} E + P$	$e^{\mathcal{W}_{\mathcal{C}_{II}}} = e^{-\Delta\mu}$	$\pi_{\mathcal{C}_{II}} = [S]k_{+1}^0 k_{-2}^0 e^{z_1 \Delta\theta\delta F_{max}} e^{-(1-z_2)\Delta\theta\delta F_{max}}$
$\mathcal{C}_{II,R} = P + E \xrightarrow{-\delta F_{max}} E_L \xrightarrow{-\delta F_{max}} E + S$	$e^{\mathcal{W}_{\mathcal{C}_{II,R}}} = e^{-\mathcal{W}_{\mathcal{C}_{II}}} = e^{\Delta\mu}$	$\pi_{\mathcal{C}_{II,R}} = [P]k_{+2}^0 k_{-1}^0 e^{-(1-z_1)\Delta\theta\delta F_{max}} e^{z_2 \Delta\theta\delta F_{max}} = \pi_{\mathcal{C}_{II}} e^{-\mathcal{W}_{\mathcal{C}_{II}}}$

many macromolecules, by virtue of their large number of degrees of freedom with which to interact with their environment, can transduce energy from environmental fluctuations to drive chemical, electrical, or even mechanical processes.<sup>9,10</sup>

It is equally important to recognize that there is both a thermodynamic and a kinetic component for describing the effect of an external fluctuation. There can be no purely thermodynamic theory sufficient to describe a non-equilibrium steady state. Dissipation is certainly necessary for driving the formation of a non-equilibrium steady state – that observation is basically a tautology – but not sufficient.

When viewed in terms of mechanical motion of some object, the stochastic pumping mechanism has come to be known as an energy ratchet<sup>40,41</sup> and is the standard theoretical description for many synthetic molecular machines.<sup>42,43</sup> Below we discuss specifically how a stochastically pumped energy ratchet was used as the design principle for the first synthetic molecular pump.<sup>44</sup>

## 4. Synthetic stochastic pumps

A stochastic pump consists of an energy well surrounded by two barriers that interact differently with some environmental fluctuation.<sup>8,45</sup> This structure inspires a straightforward approach to designing molecules that facilitate directional transport in the form of binding from one side and releasing from the other as in the original description of fluctuation driven transport across membranes.<sup>1–3</sup>

### a. Directional threading

The first such complex<sup>46</sup> was based on a “dumbbell” shaped polymer with an electron rich 1,5-dioxynaphthalene (DNP) unit placed at the middle of the dumbbell. The DNP recognizes and binds to an electron poor cyclobis(paraquat-*p*-phenylene) tetracation (CBPQT<sup>4+</sup>). The rates for binding between CBPQT<sup>4+</sup> and DNP from the two different sides were separately set by attaching two different groups to the ends of the dumbbell, one, an electrically neutral but bulky isopropylphenyl (IPP) group, and the other a positively charged pyridinium (PY<sup>+</sup>) group. According to the principle of microscopic reversibility,<sup>47</sup> the most probable mechanism for any chemical reaction in the forward direction is the same as the most probable direction in the reverse direction. Thus, if binding of the oxidized ring CBPQT<sup>4+</sup> is more likely over the IPP group than over the positively charged PY<sup>+</sup> group, then dissociation of the CBPQT<sup>4+</sup> over the IPP group is more likely than dissociation over the PY<sup>+</sup> group. Similarly, if dissociation of the reduced ring CBPQT<sup>2(+•)</sup> over the PY<sup>+</sup> electrostatic barrier is more likely than over the IPP steric barrier, then binding is also more likely over the PY<sup>+</sup> electrostatic barrier than over the IPP steric barrier. At any fixed oxidation state there can be no directional transport irrespective of the symmetry of the barriers.

The situation is very different when the redox potential of the solution is externally caused to fluctuate. When the ring is oxidized it most likely binds, and it does so over the IPP steric barrier. When the ring is reduced, it most likely dissociates, doing so predominately over the PY<sup>+</sup> electrostatic barrier. Thus,

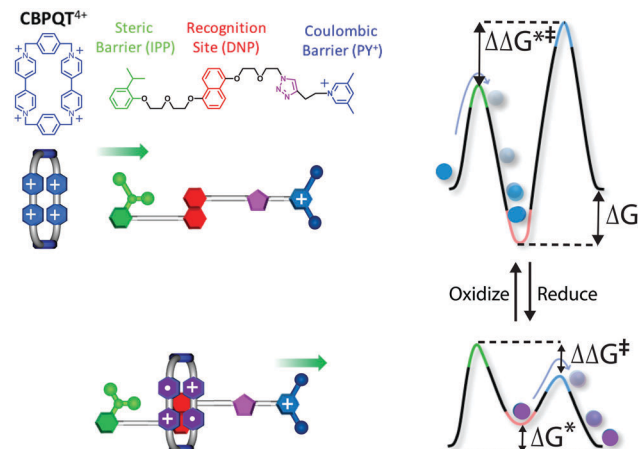


Fig. 6 Synthetic chemical implementation of a stochastically pumped energy ratchet for directional transport. The key kinetic symmetry breaking is the dependence of the height of the coulombic barrier (PY<sup>+</sup>) and independence of the steric barrier (IPP) on the redox state of the ring. The CBPQT<sup>4+</sup> is stably bound on the DNP recognition site when oxidized, but the binding is significantly weakened when the ring is reduced to the CBPQT<sup>2(+•)</sup> radical.

in a cycle of oxidation/reduction the ring will have travelled directionally over the IPP barrier to the DNP recognition site, and then over the PY<sup>+</sup> Coulombic barrier to return to the bulk.

We can use the principle of microscopic reversibility, to calculate the ratio of probabilities for “forward” threading/dethreading  $\pi_+ = \pi(\text{IPP} \rightarrow \text{DNP} \rightarrow \text{PY}^+)$  vs. “backward” threading/dethreading  $\pi_- = \pi(\text{PY}^+ \rightarrow \text{DNP} \rightarrow \text{IPP})$  based on the kinetic diagram shown in Fig. 6.

Starting, *e.g.*, in the upper left-hand corner of the mechanism in Fig. 7, there are four distinct trajectories by which the ring can bind/dissociate while following the pathway  $\rightarrow \text{IPP} \rightarrow \text{DNP} \rightarrow \text{PY}^+ \rightarrow$ , each of which has a microscopic reverse. By designing the system such that the kinetic specificities obey  $k_{-1,\text{ox}}/k_{-2,\text{ox}} \gg 1$  and  $k_{-2,\text{red}}/k_{-1,\text{red}} \gg 1$  the transitions for binding/dissociation of the reduced ring over the IPP group and for binding/dissociation of the oxidized ring over the PY<sup>+</sup> group are kinetically blocked, assuring that the system follows a zig-zag pattern. The ratio of forward to backward transitions is, in this case, well approximated as

$$\frac{\pi_+}{\pi_-} \approx e^{-\Delta G + \Delta G^*} \quad (15)$$

where  $\Delta G^*$  ( $-\Delta G$ ) is the free energy difference for binding at the DNP site when oxidized (reduced). The system shown in Fig. 6 was used to demonstrate the proof of principle that directional transport in a molecular system is possible by a stochastically pumped energy ratchet.

DFT calculations, however, show that the binding stability only changes by about  $-\Delta G + \Delta G^* \approx 4 \text{ kcal mol}^{-1}$ , not a large enough change to allow for the system to be developed to do significant work in the environment.

### b. An artificial molecular pump

A more promising interaction with regard to designing a molecular machine to harness energy from external fluctuations is the binding of CBPQT<sup>2(+•)</sup> to a (1,1'-dialkyl-4,4'-bipyridinium)

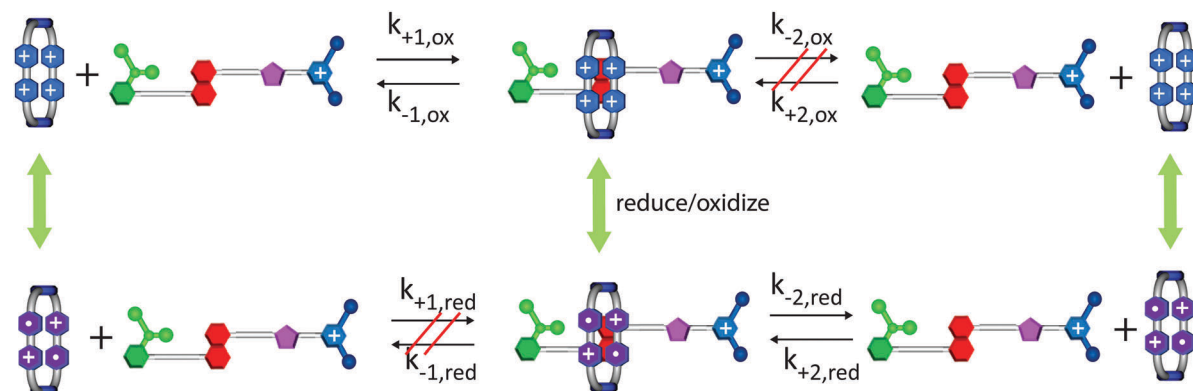


Fig. 7 Kinetic mechanism for unidirectional threading. In the oxidized state the large kinetic barrier renders the rates over the  $\text{PY}^+$  electrostatic barrier negligible, whereas when the ring is reduced the rate over the steric barrier is negligible in comparison to the rate over the electrostatic barrier. This leads to directional threading when the environment is caused to fluctuate between reducing and oxidizing conditions.

radical cation ( $\text{V}^{\bullet+}$ ). Upon oxidation of both species to  $\text{CBPQT}^{4+}$  and  $\text{V}^{2+}$ , respectively, dissociation of the complex releases nearly  $20 \text{ kcal mol}^{-1}$ , a very large amount of energy available that can be harvested to do significant work. Efforts to do just this have focussed on a structure in which an electrostatic barrier (a  $\text{PY}^+$  group) separated the bulk from a dumbbell shaped rod on which a viologen recognition site was located and that was terminated on the other end by a very bulky stopper group that would present an insuperable steric barrier to a  $\text{CBPQT}$  ring in any oxidation state. By alternately changing the redox potential of the solution between strongly reducing and strongly oxidizing a ring could be pumped onto the collecting chain with a steady state occupancy much greater than that in equilibrium at any redox potential. In this first incarnation, only one ring could be localized on the DB molecule, and this ring was only metastable – it would slowly dissociate over the  $\text{PY}^+$  when in the reduced state.<sup>48</sup> A key insight came when it was recognized that by placing a steric barrier – an IPP group between the recognition site and the collecting chain – a ring on the collecting chain could be kinetically stabilised so that several rings could be pumped onto the collecting chain.

When this design was synthesized and driven by an oscillating redox potential, it was indeed shown to be possible to pump several rings onto the collecting chain to form a non-equilibrium structure, a structure that exists at steady-state with a large probability but whose free-energy is at every instant in time significantly higher than the free energy of a ring in the bulk. This effect represents true non-equilibrium pumping<sup>49,50</sup> since the equilibrium state of the ring is “off” – the free energy of a ring in the bulk is several  $\text{kcal mol}^{-1}$  less than the free-energy of a ring on the collecting chain – at every redox potential. Several rings have been pumped onto the collecting chain, and the structure is kinetically stable for weeks after the external fluctuations have stopped. The energy profiles for both the reduced and oxidized states are shown in Fig. 8, along with schematic graphical depictions of the machine that provide the “reaction coordinate” for the motion of the rings. Traditional chemical structures of the components are also shown.

A kinetic mechanism for redox oscillation driven assembly is shown in Fig. 9, from which the forward and backward pathways described in Table 6 are evident, as are the uncoupled pathways.

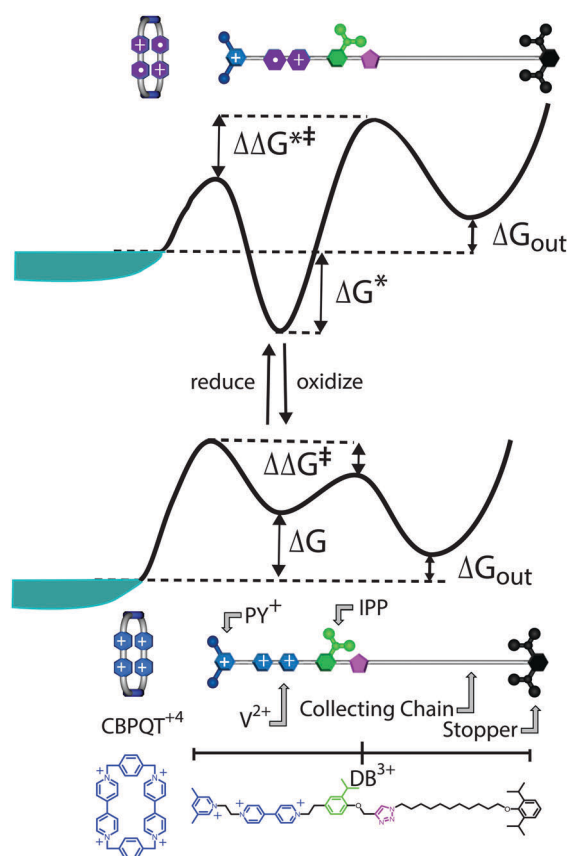


Fig. 8 Energy profiles for a  $\text{CBPQT}$  ring threading onto a  $\text{DB}$  rod for the reduced (top) and oxidized (bottom) condition. The key design feature allowing the structure to be stochastically pumped to place several rings on the collecting chain is the placement of a  $\text{PY}^+$  group on one end of the  $\text{DB}$  to serve as an electrostatic barrier by which a ring can move between the bulk and the viologen recognition site only when the ring is reduced.

The steady state concentration ratio between the assembled and disassembled oxidized states is given by

$$\frac{[\text{A}_{\text{ox}}]}{[\text{D}_{\text{ox}}]} = \frac{\pi_{\text{F}} + \pi_{\text{B}_\text{R}} + \pi_{\text{C}_\text{I}} + \pi_{\text{C}_{\text{II}}}}{\pi_{\text{F}_\text{R}} + \pi_{\text{B}} + \pi_{\text{C}_{\text{IR}}} + \pi_{\text{C}_{\text{IIR}}}} \approx e^{-\Delta G_{\text{out}}} e^{-\Delta G + \Delta G^*} \quad (16)$$

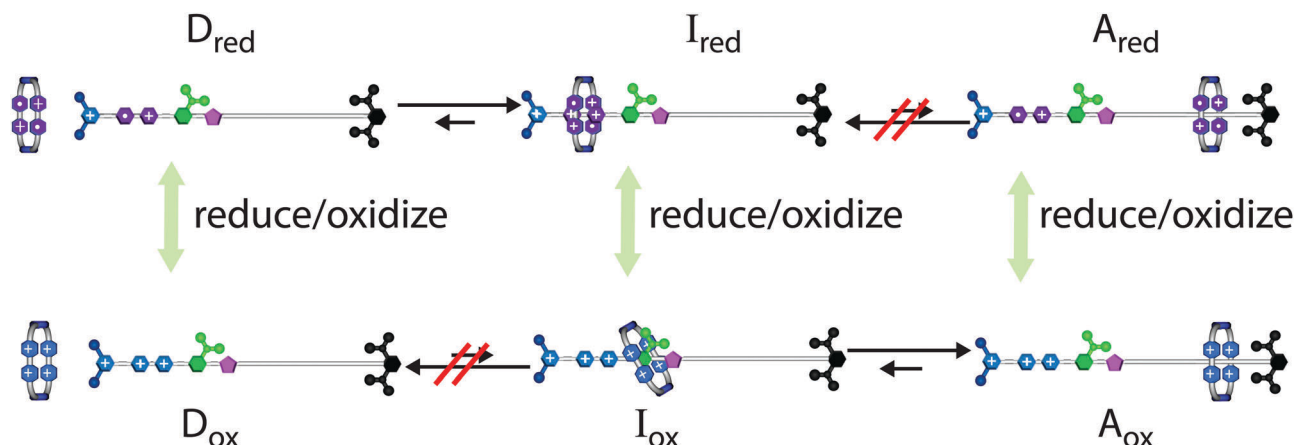


Fig. 9 Kinetic mechanism for an artificial stochastic pump. Although the equilibrium constant strongly favors  $D_{ox}$  over  $A_{ox}$  and  $I_{red}$  over  $A_{red}$  the transitions  $A_{red} \rightarrow I_{red}$  and  $I_{ox} \rightarrow D_{ox}$  are kinetically blocked by large energy barriers shown in Fig. 8. Continual cycling between oxidizing and reducing conditions pumps several rings onto the collecting chain where they are meta-stable even for a month or more.

**Table 6** Pathways between the disassembled and assembled oxidized states,  $D_{ox}$  and  $A_{ox}$ . These paths dissipate the same amount of energy  $\mathcal{W} = -\Delta G + \Delta G^*$  so in the absence of kinetic dissymmetry the fluctuations between oxidizing and reducing conditions do not lead to assembly. In addition to these coupled pathways there are two uncoupled pathways leading between  $D_{ox}$  and  $A_{ox}$ , in which the mechanical motion of the ring occurs in either the oxidized or the reduced state and for which there is no excess work

$\mathcal{F} = D_{ox} \rightarrow D_{red} \rightarrow I_{red} \rightarrow I_{ox} \rightarrow A_{ox}$	$\frac{\pi_{\mathcal{F}}}{\pi_{\mathcal{F}_R}} \approx e^{-\Delta G_{out} + \mathcal{W}}$
$\mathcal{B} = A_{ox} \rightarrow A_{red} \rightarrow I_{red} \rightarrow I_{ox} \rightarrow D_{ox}$	$\frac{\pi_{\mathcal{B}}}{\pi_{\mathcal{B}_R}} \approx e^{\Delta G_{out} + \mathcal{W}}$
$\mathcal{C}_I = D_{ox} \rightarrow I_{ox} \rightarrow A_{ox}$	$\frac{\pi_{\mathcal{C}_I}}{\pi_{\mathcal{C}_I R}} \approx e^{-\Delta G_{out}}$
$\mathcal{C}_{II} = D_{ox} \rightarrow D_{red} \rightarrow I_{red} \rightarrow A_{red} \rightarrow A_{ox}$	$\frac{\pi_{\mathcal{C}_{II}}}{\pi_{\mathcal{C}_{II R}}} \approx e^{-\Delta G_{out}}$

where the second expression arises from taking the approximation that the kinetic blockade is very strong, as is the case experimentally.

With this explicit formulation, we see clearly the importance of the kinetic asymmetry on which stochastic pumping is based. If  $\pi_{\mathcal{B}} > \pi_{\mathcal{F}}$  the fluctuations favor disassembly relative to the equilibrium case. Only when  $\pi_{\mathcal{F}} > \pi_{\mathcal{B}}$  do the fluctuations favor assembly. Both  $\mathcal{F}$  and  $\mathcal{B}$  obey Jenck's "rule" for vectorial coupling<sup>51</sup> according to which the chemical steps of reduction/oxidation are interleaved with the mechanical steps in which the ring undergoes translational motion relative to the DB rod. Selection between the  $\mathcal{F}$  and  $\mathcal{B}$  pathways can be achieved through allosteric interactions.<sup>52</sup>

Note that once the system has reached steady state the assembled dumbbell – ring complex no longer dissipates energy. Because of the large kinetic barrier preventing the ring from slipping over the steric barrier to the recognition site the ring does not usually back up to the intermediate state in either the oxidized or reduced states, assuring that the assembled structure is stable for weeks if not months even after the redox oscillation is turned off. It is just not the case that the system

adapts to a continuously dissipate energy. Instead, an intelligently designed molecular machine follows assembly pathways in which, in the presence of fluctuations, much more energy is dissipated in the assembly than in the disassembly, and once assembled the structure is stable without further significant dissipation.

There are numerous other examples of what is termed "dissipative self-assembly".<sup>53–55</sup> Most involve situations in which the precursor molecules switch between forms in which assembly is thermodynamically favored and forms in which the assembly is disfavored. The switching between these two forms involves catalytic conversion of a chemical fuel from a high-energy substrate to a lower-energy product, with the difference in energy being dissipated in the environment. In some cases the energy is provided by absorption and dissipation of optical energy. It should be noted that the fundamental constraints on the kinetics of the system are very different for light driven *vs.* chemical catalysis driven systems. The "dissipative assembly" systems are very interesting examples of energy driven processes, but the assembled structures are not non-equilibrium structures in the sense that the CBPQT/DB complex  $A_{red}/A_{ox}$  are non-equilibrium structures. The assembly of the pump shown in Fig. 9 is not thermodynamically favored relative to the disassembled form at any redox potential whatsoever. The cycling between high and low redox potential provides the energy. In contrast, in the so-called dissipative assembly systems, the monomers cycle between a state in which assembly is favored and a state in which disassembly is favored.

In the recent paper by Boekhoven *et al.*<sup>54</sup> a monomeric building block with a carboxylate group reacts with a chemical fuel, DMS, to form a methyl ester that self assembles into a fibrous structure. Both the assembled and monomeric methyl ester containing molecule can undergo ester hydrolysis to regenerate the carboxylate which would disassemble. Ester hydrolysis is not the microscopic reverse of the reaction with DMS that forms the methyl ester, and so significant energy is released in each cycle of methyl ester formation and ester hydrolysis.

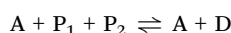


The assembled form is not a “non-equilibrium” structure – the methyl ester containing monomers spontaneously assemble. The energy released in the chemical cycle is used for control of the resulting very rich dynamical behavior.

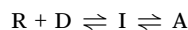
The stochastically pumped CBPQT/DB complex in Fig. 8 and 9 performs one important function of life – it uses energy from fluctuations to recruit material (rings) from the environment to increase its own complexity,<sup>56</sup> and sequesters that material onto a compartment that is separate from the bulk on the collecting chain. So far, however, the system has not been designed to self-replicate – to make more copies of itself. Self-replication is another hallmark of living systems, and allows for what Pross has termed “dynamic kinetic stability”.<sup>57</sup> We can at least entertain this possibility by thinking of the mechanism by which the DB rod in the example above is made.

### c. Toward self-replication and systems chemistry

The functional  $\text{PY}^+-\text{V}^{2+}\text{-IPP} = \text{P}_1$  unit is linked to the collecting chain stopper unit ( $\text{CC-S} = \text{P}_2$ ) by a copper ion catalyzed azide alkyne click reaction to form a triazole ring linking the two components together. One approach to self-replication would be to design the chemistry such that a ring in the assembled state, but not in the bulk could catalyze the linkage reaction



Coupled with the assembly scheme



these two reactions form an autocatalytic loop. At any fixed redox potential, there will be a very small amount of the assembled complex, and hence a very small rate of catalysis of the formation of D. When the redox potential is caused to fluctuate, however, the ring is stochastically pumped onto the collecting chain where it becomes catalytically active and the reaction will rapidly take off to use up all of the precursor materials R,  $\text{P}_1$ ,  $\text{P}_2$  to form A. The tremendous challenge, of course, is to figure out precisely by what chemistry the environment on the collecting chain can switch the catalytic activity of the ring R.

One of the great challenges of contemporary chemistry is to understand how complex matter arises from simple chemicals.<sup>54</sup> The fluctuation driven self-assembly of the CBPQT ring – DB complex in Fig. 8 and 9 is one example, but recent work highlights systems of simple components that have imbued in their structures remarkable flexibility by which they can organize into many different complex structures.<sup>58–60</sup>

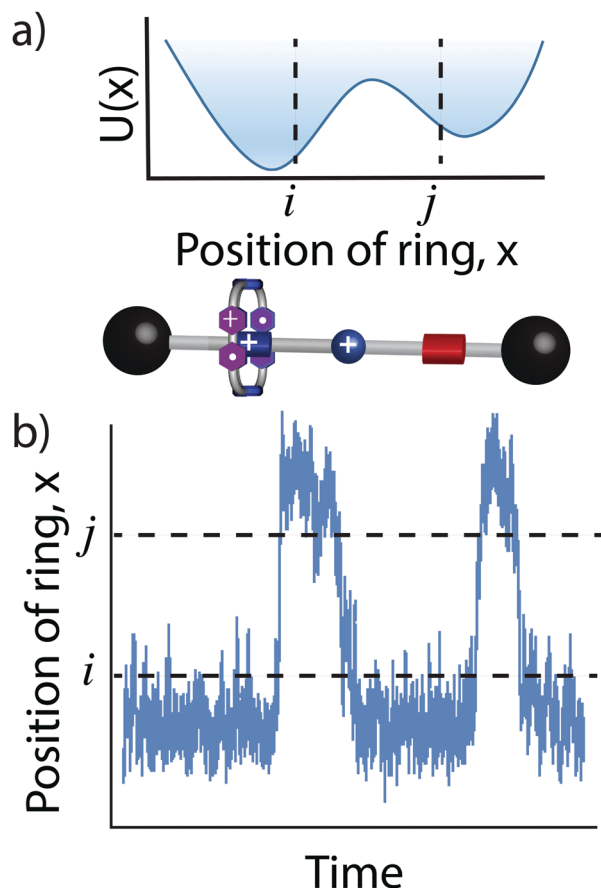
### d. Equilibrium or not equilibrium, that is the question

Progress toward elucidation of how complex matter arises from simpler molecules in the presence of energy driven fluctuations will ultimately come from collaboration between synthetic chemists, physical chemists, and theoretical physicists all willing to roll up their sleeves and confront molecules in their native environments. One important realization stemming from consideration of the environment of molecules in solution is that, while the ensembles of states and trajectories of molecular machines

may be “far from equilibrium” *i.e.*, not conforming to a Boltzmann distribution, an individual molecular machine is, in the only sense meaningful for an individual entity, very, very close to mechanical equilibrium. When we refer to the assembled molecules  $\text{A}_{\text{ox}}$  and  $\text{A}_{\text{red}}$  in Fig. 9 as non-equilibrium “structures” we recognize that these configurations would be present at equilibrium, perhaps only one molecule out of a million, but still present. Further, the physical movements by which the synthetic pump undergoes transition between different states are processes that occur also at equilibrium, and are equilibrium motions. This recognition registers an important observation – the theoretical formulation to describe stochastic processes formulated by Onsager and Machlup<sup>32</sup> is appropriate for the description of individual molecular machines even in environments that are “far from thermodynamic equilibrium”. The knee-jerk reaction of many scientists is that the Onsager–Machlup<sup>32</sup> theory is valid only “near equilibrium in the linear regime”. This restriction is a perfectly true, but very misleading expression of the conditions necessary for application of the Onsager–Machlup theory. To appreciate why this is the case we must read Onsager and Machlup’s own words<sup>32</sup> – “The essential physical assumption about the irreversible processes is that they are linear, *i.e.*, that the fluxes are proportional to the forces that cause them.” At the single molecule level, this requirement is nothing other than the statement that the relevant motions must be dominated by viscous friction rather than by inertia – *i.e.*, that the motions are characterized by a low Reynold’s number,<sup>61</sup> a condition that holds for all molecules in solution. The conditions necessary for application of Onsager and Machlup’s stochastic thermodynamic theory are, at least in molecular science, ubiquitous even when the system is far from thermodynamic equilibrium.<sup>62</sup>

Consider a single molecule of the molecular switch shown in Fig. 1. The potential energy profile (Fig. 10a) as a function of position of the ring on the DB rod can be calculated using density functional theory (DFT), and the ring can be modelled as a “Brownian” particle moving on that potential, with its motion described by a Langevin equation. One realization of the motion, with two back and forth transitions between the recognition sites shown in Fig. 10b.

The transitions can be broken into “waiting times” in which the ring undergoes Brownian motion near one interaction site, and a barrier interaction time in which the ring makes a “hop” from one site to the other. This transition, or barrier interaction, time is very much shorter than the lifetime on one or the other recognition sites. We might imagine that if the barrier were made steeper, *e.g.* either by increasing the size of a steric barrier, or the charge on an electrostatic barrier, between the two recognition sites, that the barrier interaction time would be longer. In fact, the barrier interaction time is shorter when the barrier is steeper. The waiting time, on the other hand, increases exponentially with the height of the barrier. Although the decrease in barrier interaction time on increasing the barrier height may seem counterintuitive to anyone with experience walking in the mountains, the result is an easily derived corollary of the principle of microscopic reversibility.<sup>27</sup> The most probable



**Fig. 10** (a) Energy profile  $U(x)$  as a function of the position of a CBPQT<sup>2(+)</sup> along a DB shaped rod with two recognition sites (see Fig. 1 and discussion) with two points,  $i$  and  $j$ , indicated for reference. (b) Brief Langevin simulation capturing two back and forth transitions between the two recognition sites. There are generally many re-crossing events over  $i$  and  $j$  before that trajectory in which the ring touches for the last time  $i$  before reaching  $j$ , or touches  $j$  for the last time before reaching  $i$ .

trajectory from any point “ $i$ ” to a different point “ $j$ ” is the microscopic reverse of the most probable trajectory from point “ $j$ ” to point “ $i$ ”.<sup>27,47</sup>

We can focus on what is known as the “last touch first touch” (LTFT) time – that time between when a ring last touches point “ $i$ ” and first touches point “ $j$ ” and prove<sup>27</sup> the remarkable relation

$$\langle \tau_{\text{LTFT}}(i \rightarrow j) \rangle = \langle \tau_{\text{LTFT}}(j \rightarrow i) \rangle \quad (17)$$

an identity that holds for any points  $i$  and  $j$  irrespective of the potential energy surface.

Further, the statistical properties of the ensembles of forward and microscopic reverse trajectories are tied by symmetry relations from which the very general equality for the ratio of the conditional probabilities for a forward and backward transition at any time  $t$  is derived<sup>27</sup>

$$\frac{\pi\left(i \xrightarrow{S[t]} j\right)}{\pi\left(j \xrightarrow{S^\dagger[t]} i\right)} = e^{\Delta G_{ij}^0} e^{\mathcal{W}_{S,ij}} \quad (18)$$

where  $\mathcal{W}_{S,ij} = \sum \delta F(t) \Delta \theta_{ij}$  is the excess work – the difference between the work exchanged with the environment under the conditions pertaining at the moment  $t$  at which the transition occurs and the work  $\Delta G_{ij}^0$  that would be exchanged under some fixed reference condition. The summation sign indicates that there could be lots going on – the pH and redox potential might both be different than the reference value, the temperature and pressure might also be different than the reference value, and the chemical potential of some fuel substrate may be different (and time varying) than that of the waste product. Eqn (18) is identical to eqn (7) except that here we explicitly denote that the excess work depends on the time at which the transition occurs, and we also recognize that there could be more than one source of external energy for the process. The simplicity of eqn (18) results from a separation of time scales where it is recognized that the barrier interaction time is so short that any “hop” from one state to another is accomplished at what is effectively a constant value for any  $\delta F(t)$ . The net transition probabilities can be integrated to obtain the average of the ratio of the probabilities for the forward and backward transitions.

$$\frac{\int p(i) \pi\left(i \xrightarrow{S[t]} j\right) dt}{\int p(j) \pi\left(j \xrightarrow{S^\dagger[t]} i\right) dt} = e^{\Delta G_{ij}^0} \frac{\int p(i) \pi\left(j \xrightarrow{S^\dagger[t]} i\right) e^{\mathcal{W}_{S,ij}} dt}{\int p(j) \pi\left(j \xrightarrow{S^\dagger[t]} i\right) dt} \quad (19)$$

where eqn (18) was used to write  $\pi\left(i \xrightarrow{S[t]} j\right)$  in terms of  $\pi\left(j \xrightarrow{S^\dagger[t]} i\right)$ . Setting the ratio to unity we arrive at the steady state condition

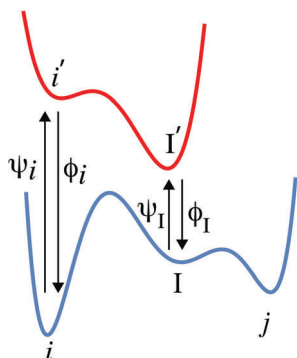
$$\frac{p(j)}{p(i)} \Big|_{ss} = e^{\Delta G_{ij}^0} \langle e^{\mathcal{W}_{S,ij}} \rangle \quad (20)$$

Eqn (17)–(20) are not “new and novel” non-equilibrium relations, but rather relations derived based on recognition that, even in the presence of non-equilibrium fluctuations, or of external driving, or of chemical potential differences of a substrate and product, the transitions of an individual molecular machine are equilibrium processes.<sup>31,43</sup> Eqn (17)–(20) are corollaries of the principle of microscopic reversibility.<sup>27</sup>

Another important point registered by recognition that the motions by which chemical systems adapt to fluctuations in their environment occur at low Reynold’s number is that physical resonances below microwave frequency are impossible. Attempts to analogize adaptation by chemical transitions with the shattering of glass by acoustic waves or other resonant phenomena is very misleading. It has long been known that the response of a chemical network to time dependent fluctuations can be a non-monotonic function of frequency,<sup>8,19</sup> but the “peaks” are described in terms of “windowing” in which the response is maximized between two relaxation times of the chemical reaction network and arise from dispersive effects, the antithesis of resonance.

### e. Beyond Boltzmann

Non-equilibrium steady states can be driven by the action of many different types of fuel – light, external modulation of the



**Fig. 11** Potential energy surface model for a proton pump with an intermediate state  $I$  between states  $i$  and  $j$ . In the discussion by Warshel the transitions between the blue and red energy surfaces explicitly involved photochemical processes. Here, we treat separately the case of photochemical driving and of driving by chemical catalysis and show that while a non-equilibrium steady state can result from either chemical or photochemical driving, the design principles for using these two fuels are very different.

generalized forces (pH, redox potential, pressure, *etc.*) in Table 2, or catalysis of the conversion of chemical fuel to a waste product. For example, ATP hydrolysis powers many biological motors. It is very tempting to write and speak in terms of a generic energy input. The failure to specify the details of the energy input, however, can be very misleading.<sup>63</sup> The transitions for absorption and emission of light obey the Einstein relations according to which the conditional probability to absorb a photon and undergo a transition from a low energy to a high energy state is identical to the conditional probability to interact with a photon and undergo a transition from a high energy state to a low energy state concomitant with stimulated emission of a photon. In contrast the transitions for chemical catalysis of even an extremely exergonic reaction must obey microscopic reversibility. Microscopic reversibility requires that thermal activation from a low energy to a high energy state is exponentially less likely than transition from a high energy to a low energy state. Let us examine a specific case based on a model for a proton pump<sup>64</sup> involved shown in Fig. 11.

The ratio of forward and reverse rate constants for the individual transitions on either the red or blue potential are given by Boltzmann expressions

$$\frac{k_{i \rightarrow I}}{k_{I \rightarrow i}} = e^{G_i - G_I}; \quad \frac{k_{I \rightarrow j}}{k_{j \rightarrow I}} = e^{G_I - G_j}; \quad \text{and} \quad \frac{k_{I' \rightarrow I}}{k_{I \rightarrow I'}} = e^{G_{I'} - G_I} \quad (21)$$

There are two paths from  $i$  to  $j$  and of course two returning from  $j$  to  $i$ . The ratio of the net probabilities is

$$\frac{\pi\left(i \xrightarrow{S_1[t]} j\right) + \pi\left(i \xrightarrow{S_2[t]} j\right)}{\pi\left(j \xrightarrow{S'_1[t]} i\right) + \pi\left(j \xrightarrow{S'_2[t]} i\right)} \quad (22)$$

$$= \frac{p(i)k_{I \rightarrow j}[\psi_i p(i')k_{I' \rightarrow I'} p(I')\phi_I + k_{i \rightarrow I}]}{p(j)k_{j \rightarrow I}[\phi_i p(i')k_{I' \rightarrow I'} p(I')\psi_I + k_{I \rightarrow i}]}$$

If the barrier between states  $i$  and  $I$  is very large the term  $k_{i \rightarrow I}$  can be neglected in the numerator, and the term  $k_{I \rightarrow i}$  can be

neglected in the denominator. This simplification allows cancellation of the factors  $p(i')$  and  $p(I')$ . By setting the resulting ratio to unity – the steady state condition – we solve for the steady state ratio to find

$$\frac{p(j)}{p(i)} \Big|_{ss} = \left[ \frac{\psi_i \phi_j}{\phi_i \psi_j} e^{G_I - G_i} e^{G_{I'} - G_{I'}} \right] e^{G_i - G_j} \quad (23)$$

For a photochemical process the transitions between the red and blue energy surfaces are governed by the Einstein relations

$$\frac{\psi_{i,I}}{\phi_{i,I}} = \frac{\rho(\nu_{i,I})}{\frac{8\pi h \nu_{i,I}^3}{c^3} + \rho(\nu_{i,I})} \quad (24)$$

Where,  $\nu_i = |G_i - G_{I'}|/h$  and  $\nu_I = |G_I - G_{I'}|/h$ ,  $h$  is Planck's constant, and  $\rho(\nu_{i,I})$  is the photon density at the frequency  $\nu_{i,I}$ . If we model the light source as a black-body radiator the expression in brackets in eqn (23) is unity if the temperature of the black-body is the same as that of the chemical system, i.e., the steady-state distribution is the equilibrium distribution. In the limit that the temperature of the black-body is very large

however, the factor  $\frac{\psi_i \phi_j}{\phi_i \psi_j} \rightarrow 1$  and the expression in brackets

that modifies the equilibrium Boltzmann factor  $e^{G_i - G_j}$  is  $[e^{G_I - G_i} e^{G_{I'} - G_{I'}}]$ . The design principle is clear – in order to construct an effective photochemical pump it is important to construct a molecule which after transition to the excited state surface dissipates a large amount of energy in the transition  $i' \rightarrow I'$ . That transition can very reasonably be described as a power stroke, and the overall mechanism can be described either as an energy ratchet, or as a power-stroke mechanism since the factor that modifies the equilibrium constant depends on the energy released in the transition  $i' \rightarrow I'$ .

The situation is very different when the system is driven by chemical catalysis of an exergonic reaction  $S \rightarrow P$  where each conversion from  $S$  to  $P$  releases  $\Delta\mu$  energy. In this case, the blue and red surface can be thought of as the energy profiles governing the conformational dynamics of an enzyme in the “free” and “bound” state, respectively. The transitions between the two surfaces can be broken into two processes. One process describes the binding and dissociation of  $S$ , with rate constants  $f_{i,on}$ ,  $f_{i,on}$  and  $f_{i,off}$ ,  $f_{I,off}$ , respectively. The other process describes the binding and dissociation of  $P$ , with rate constants  $g_{i,on}$ ,  $g_{I,on}$  and  $g_{i,off}$ ,  $g_{I,off}$ , respectively. The important ratio of the net transition rates between the two surfaces can be written

$$\frac{\psi_i \phi_I}{\phi_i \psi_I} = \frac{(f_{i,on} + g_{i,on})(f_{I,off} + g_{I,off})}{(f_{i,off} + g_{i,off})(f_{I,on} + g_{I,on})} \quad (25)$$

There are two relationships between the  $f$ 's and  $g$ 's that can be used to simplify eqn (25). The first is the expression for the equilibrium constant for conversion between  $S$  and  $P$  in any fixed state

$$\frac{f_{i,on} g_{I,off}}{g_{i,on} f_{I,off}} = \frac{f_{i,on} g_{I,off}}{g_{i,on} f_{I,off}} = e^{\Delta\mu} \quad (26)$$

**Table 7** Summary of the effect of the kinetic factor given as the ratio of “off” rates and of the thermodynamic factor the magnitude of the factor in brackets in eqn (28) and on the chemical dissipation due to reaction between S and P

	$\Delta\mu < 0$	$\Delta\mu = 0$	$\Delta\mu > 0$
$\frac{f_{i,\text{off}}}{f_{i,\text{on}}} \frac{g_{i,\text{off}}}{g_{i,\text{on}}} < 1$	$[\dots] < 1$ dissipation	$[\dots] = 1$ no dissipation	$[\dots] > 1$ dissipation
$\frac{f_{i,\text{off}}}{f_{i,\text{on}}} \frac{g_{i,\text{off}}}{g_{i,\text{on}}} = 1$	$[\dots] = 1$ dissipation	$[\dots] = 1$ no dissipation	$[\dots] = 1$ dissipation
$\frac{f_{i,\text{off}}}{f_{i,\text{on}}} \frac{g_{i,\text{off}}}{g_{i,\text{on}}} > 1$	$[\dots] > 1$ dissipation	$[\dots] = 1$ no dissipation	$[\dots] < 1$ dissipation

The second relation recognizes that the ratio of clockwise to counterclockwise rate constants for a cycle in which no work is dissipated in the environment is unity and allows us to write

$$\frac{f_{i,\text{on}} f_{i,\text{off}}}{f_{i,\text{off}} f_{i,\text{on}}} = \frac{g_{i,\text{on}} g_{i,\text{off}}}{g_{i,\text{off}} g_{i,\text{on}}} = e^{-(G_i - G_i)} e^{-(G_i' - G_i')} \quad (27)$$

When we insert these thermodynamic identities into eqn (25), and the result of that operation into eqn (23) we find

$$\frac{p(j)}{p(i)} \Big|_{\text{ss}} = \left[ \frac{\left(1 + \frac{f_{i,\text{off}}}{g_{i,\text{off}}} e^{\Delta\mu}\right) \left(1 + \frac{f_{i,\text{off}}}{g_{i,\text{off}}}\right)}{\left(1 + \frac{f_{i,\text{off}}}{g_{i,\text{off}}}\right) \left(1 + \frac{f_{i,\text{off}}}{g_{i,\text{off}}} e^{\Delta\mu}\right)} \right] e^{G_i - G_j} \quad (28)$$

The sign of the term in brackets is determined by  $\Delta\mu$  and by the ratio  $\frac{f_{i,\text{off}}}{g_{i,\text{off}}} \frac{g_{i,\text{off}}}{f_{i,\text{off}}}$  as summarized in Table 7.

The ratio of the “off” rates  $\frac{f_{i,\text{off}}}{g_{i,\text{off}}} \frac{g_{i,\text{off}}}{f_{i,\text{off}}}$  that determines whether the term in brackets that modifies the Boltzmann expression for the steady state ratio  $\frac{p(j)}{p(i)} \Big|_{\text{ss}}$  is independent of the free energies of the states. Thus even though there may be a “power-stroke” the mechanism by which chemical catalysis drives the formation of a non-equilibrium steady state, including a steady state where there is a net directional motion, is not a “power-stroke” mechanism. Instead, all molecular machines driven by chemical catalysis operate as information ratchets.

The energy profiles in Fig. 11, and the rate constants  $f$  and  $g$  are very similar conceptually to the model first described by Andrew Huxley for the motion of myosin on actin.<sup>65</sup> When Huxley’s mechanism is reexamined in light of microscopic reversibility<sup>66</sup> using eqn (26) and (27), the major achievement of that model – the reproduction of the experimentally observed force vs. velocity curves – is maintained. The rederived theoretical expressions however make very different predictions about how the behavior would change if the energy released in the power-stroke could be changed. This experimental manipulation of the power stroke has not been accomplished for myosin or any other biomolecular machine. A synthetic molecular machine powered by catalysis of a chemical reaction has been recently reported.<sup>67</sup> This machine works by an information ratchet mechanism,<sup>31,40,68</sup> not by a power-stroke or energy ratchet mechanism.

This result demonstrating the impossibility of a power-stroke mechanism for a molecular motor driven by catalysis

of a chemical reaction is general and based on the principle microscopic reversibility.<sup>30–32</sup> All motors driven by chemical catalysis, including ATP hydrolysis driven biomolecular motors, function as information ratchets. In contrast, Motors driven by external changes in the chemical composition of the medium, or by pulses of chemical fuel, can be driven by a power-stroke or energy ratchet mechanism, as demonstrated in several recent papers.<sup>69,70</sup> External chemical perturbations function more similar to light than to chemical catalysis in this regard.

## 5. Conclusions

The equation

$$\frac{[P]}{[S]} \Big|_{\text{ss}} = K_{\text{eq}} \left\langle e^{\Delta\theta[\delta F(t') - \delta F(t'')]} \right\rangle \quad (29)$$

that is the cornerstone of the stochastic pumping model of molecular adaptation and free-energy transduction<sup>8,9</sup> is both striking and disappointing. The equation is striking because, in a compact form, it relates non-equilibrium steady-state concentrations to thermodynamic quantities – the equilibrium constant and the exponential average of the work exchanged between the external fluctuations and the environment in converting substrate to product. The equation is disappointing because the exponential average of the work exchanged is not a state function and depends on the kinetic details of the many possible paths for the interconversion between substrate and product. Any hope of being able to capture the non-equilibrium steady state in terms of some thermodynamic variational principle is out the window.

The sign of the quantity  $\ln(\langle e^{\Delta\theta[\delta F(t') - \delta F(t'')]} \rangle)$  that determines whether S or P is favoured is determined by the character of the energy barriers. This was emphasized by Astumian and Robertson<sup>8</sup> by focussing on a situation with an enzyme where the bound form that interacts with the fluctuation was a true steady-state intermediate with a very small concentration. Even so, the enzyme absorbs energy to drive formation of a non-equilibrium steady state, and the steady state favours either substrate or product depending solely on the interaction between the external fluctuation and the kinetic barrier heights. Pross<sup>57</sup> and Pascal and Pross<sup>71</sup> have also emphasized the importance of kinetics for molecular adaptation, where they focus on self-replication as a means of attaining dynamic kinetic stability.

When, in a fluctuating environment, a chemical system can “choose” between a macroscopic state with some dissipation



and with kinetic asymmetry, or a macroscopic state with much dissipation but no kinetic asymmetry (see the TOC graphic), the system self-selects the state with kinetic asymmetry according to the mechanism known as stochastic pumping. The extent of the selectivity depends on the difference between the average dissipation in the forward and reverse directions. It is an inconvenient truth that the effect of external fluctuations on chemical reactions (and other physical processes) depends on the entire trajectory, and especially on the energy barriers (transition states), along the path leading between the two states and not only on the properties of the beginning and end states.

This path dependence does admit the possibility of very rich and complex behavior. The effect of an external fluctuation is described in terms of an external control parameter,  $F_i$  and an internal response function  $\theta_i$ . Thus, a “pot” of precursor molecules exposed to a fluctuating redox potential could self-assemble to an entirely different structure than that formed when the same “pot” of molecules is exposed to temperature fluctuations, even when the dissipation is identical.

Computational kinetic models may well be very useful in guiding experimentalists in exploring fluctuation driven assembly of complex structures. A word of warning is necessary however. The rate constants in any computation must be assigned in consistency with either the Einstein relations for photochemical processes, or with microscopic reversibility for thermally activated processes.<sup>63</sup> It is all too easy to fool oneself, and others, by studying toy kinetic models with rate constants assigned for mathematical convenience rather than physical consistency. A cautionary tale is found in work on the appearance of homo-chirality, an important question in the study of the origin of life. Biological organisms on earth are comprised mainly of L-amino acids even though there is no energy difference between L- and D-isomers. How did this asymmetry come about? Several theoretical studies focused on so-called re-cycling models accompanied by auto-catalysis. The kinetic mechanisms end up being rather complicated so a common approach is to ignore many back reactions, and then assign rate constants *ad libitum*. When the resulting equations are solved, it appears that weak fluctuations can result in the evolution of a system in which the concentration of one isomer increases at the expense of the other isomer. Unfortunately, these models have a fundamental flaw – the rate constants are not consistent with the principle of microscopic reversibility. Blackmond<sup>30</sup> has evocatively described kinetic models that ignore the principle of microscopic reversibility as “if pigs fly” chemistry. The last thing the field of systems chemistry needs is flying pig models of fluctuation driven assembly and adaptation, which is a very real and present danger with models of the evolution of toy chemical networks that use *ad hoc* rules for describing generic external drives acting on “active matter”.

There has also been a tremendous over-emphasis of the term “dissipation” or “entropy production” in the recent literature. The operation of a molecular machine, or the maintenance of a non-equilibrium steady state, or the creation of a structure that, in equilibrium would exist only in minute amounts, of course requires the expenditure of energy which is ultimately dissipated

in the environment thereby producing entropy. Dissipation alone, however, is not sufficient for any of these functions. The only thing that dissipation of energy in and of itself can accomplish is heating. The *sine qua non* feature that allows external energy to be used to create complex structure,<sup>56</sup> or to be transduced by macromolecules into the performance of useful work,<sup>42,43</sup> is kinetic asymmetry.<sup>8,9</sup>

The recent proposal of “dissipative adaptation”, which seems to be based, at least pictorially,<sup>37,38</sup> on a barrier hopping model that is strikingly like the model used to illustrate the mechanism of stochastic pumping,<sup>8,72</sup> focuses on dissipation but ignores the kinetic asymmetry of the system by which the chemical transitions absorb and dissipate more energy when undergoing transition in one direction than in the other. It is the kinetic asymmetry, as emphasized in the stochastic pump theory<sup>8,9</sup> that provides directionality for biomolecular machines and synthetic molecular motors, and which allows chemical systems to adapt to their environment. The behavior of the model system in eqn (9) is understandable in terms of the “Brownian Motor Principle”<sup>73</sup> according to which mechanisms for preventing undesired motion are as or more important than mechanisms for causing the desired motion. The reason that fluctuations favor state B in the example given in eqn (9) is that once the molecule converts to state B', unlike conversion to A', the return is kinetically blocked. It is simply impossible to understand how fluctuations influence the evolution of a chemical system without consideration of the kinetics as well as of the thermodynamics of chemical systems.<sup>57</sup>

The design and synthesis of molecular machines has recently been recognized by the award of the Nobel Prize in Chemistry to Ben Feringa,<sup>74</sup> Jean-Pierre Sauvage,<sup>75</sup> and Fraser Stoddart.<sup>76</sup> Although the push toward application of these remarkable devices remains in its infancy<sup>77</sup> there has already been important insight provided into the fundamental aspects of the principles by which molecules can be designed to use energy input from the environment to carry out mechanical tasks,<sup>78</sup> including self-assembly of complex structures from simpler starting materials.<sup>13,79</sup> It can only be expected that by using mechanically bonded molecules<sup>21</sup> as a springboard, continued collaboration between scientists in many different disciplines will lead to a rapidly evolving understanding of the fundamental principles of energy driven self-organization of complex matter and the role of energy driven fluctuations in the origin of life.<sup>80</sup>

## Conflicts of interest

There are no conflicts to declare.

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