# Kinetic asymmetry and the directionality of non-equilibrium molecular systems

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Scientists have long been fascinated by the biomolecular machines in living systems that process energy and information to sustain life. The first synthetic molecular rotor capable of performing repeated 360° rotations due to a combination of photo- and thermally activated processes was reported in 1999. The progress in designing different molecular machines in the intervening years has been remarkable, with several outstanding examples appearing in the last few years. Despite the synthetic accomplishments, there remains confusion regarding the fundamental design principles by which the motions of molecules can be controlled, with significant intellectual tension between mechanical and chemical ways of thinking about and describing molecular machines. A thermodynamically consistent analysis of the kinetics of several molecular rotors and pumps shows that while light driven rotors operate by a power-stroke mechanism, kinetic asymmetry – the relative heights of energy barriers – is the sole determinant of the directionality of catalysis driven machines. Power-strokes – the relative depths of energy wells – play no role whatsoever in determining the signo f the directionality. These results, elaborated using trajectory thermodynamics and the nonequilibrium pump equality, show that kinetic asymmetry governs the response of many non-equilibrium chemical phenomena.

Entry for the Table of Contents
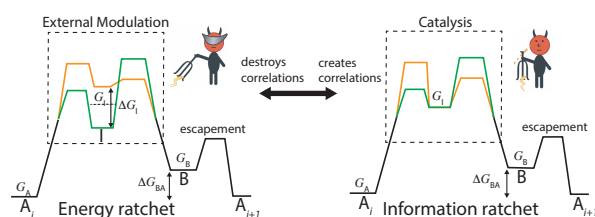
Ratchet mechanisms allow external energy to drive pumping or directional motion on the ground state. An energy ratchet uses external modulation to destroy correlations present at equilibrium and an information ratchet uses catalysis and allosteric interactions to create correlations between the energy profile and occupancy of a binding site. A key design principle is kinetic asymmetry - a difference in barrier heights in the two states of the energy profile.

1. Introduction

Molecular motors are essential for life[1]. They use metabolic (chemical) energy to power necessary tasks in cells ranging from intra- and intercellular transport, establishing ion gradients essential for action potentials of nerves, and for synthesis of adenosine triphosphate (ATP), the energy currency of the cell[2]. Biomolecular motors can also be powered by light. Recently rhodopsin molecules were shown to undergo repeated light-driven rotation[3]. Feringa and colleagues reported the first synthetic molecular rotor capable of performing repeated 360° rotations[4] in 1999, and progress in designing different molecular machines in the intervening years has been remarkable[5-7], with several excellent review[8-18] articles describing this progress. Outstanding recent examples of molecular rotors include ones driven by light[19], by electricity or modulation of the redox potential[20], and by chemical catalysis[21,22]. Progress has also been rapid in the field of molecular pumps[23-25] that can use light[26,27], electricity and/or redox modulation[28,29], and chemical catalysis[30] to create and maintain concentration gradients. Further, both DNA rotors[31] and walkers[32] as well as small molecule walkers[33-35] that can move directionally along a track have been synthesized, and efforts have begun to incorporate individual molecular machines in larger structures[36-39]. Indeed, it appears that the field is on the cusp of the type of breakthrough advances that led to the incursion of electric motors, invented in the early 19th century, into every aspect of modern life starting in the late 19th and early 20th centuries.

Despite the synthetic accomplishments, there remains confusion in the field regarding the fundamental design principles[40,41] by which the motions of molecules can be controlled. Further there is significant controversy regarding even what constitutes a molecular machine[42] and whether the chemicals that provide the energy should be termed fuel[43] and waste in analogy to the gasoline that powers our automobiles or described as substrate[44] and product in consistency with terminology for reactions facilitated by enzymes and other catalysts. Finally, it must be acknowledged that some of the theory purporting to describe molecular machines is misleading regarding the respective roles of thermodynamics vs kinetics in controlling the limiting behavior of molecular machines. Much work on thermodynamics of molecular machines has been based on the implicit idea that the engine design is optimized, and hence only thermodynamic limits are relevant. This is a reasonable assumption for many, but not all, biomolecular motors. Synthetic molecular motors, on the other hand, are certainly not optimized, and in order to make progress it is essential to understand the key design principle of kinetic asymmetry[45,48] by which input of directionless chemical energy can be harvested by a molecular machine to produce a directional response or to pump and maintain a system out of equilibrium.

Some early work focused on understanding how time dependent external electric fields can allow energetically uphill transport of a substance mediated by a transmembrane pump[46-49]. The electrical properties of the enzyme provide the thermodynamic means to input energy, and the different dipole moments of the transition states provide the kinetic asymmetry[47,48] that specified the direction of pumping. By intelligent design in the case of synthetic molecular machines, or by evolution in the case of biomolecular machines, the kinetic asymmetry[48] can be manipulated to favor either side of the membrane at steady-state according to the non-equilibrium pumping equality[49]

$$(\mu_{\text{in}} - \mu_{\text{out}})_{\text{ss}} = RT \ln\langle e^{\mathcal{W}_S/RT} \rangle$$

where The sign of $\ln\langle e^{\mathcal{W}_S/RT} \rangle$ is determined by the effect of the applied perturbation on the kinetic barriers – the kinetic asymmetry – leading to the conclusion[49]

> "An enzyme can be designed, either through protein engineering or by evolution, to couple a fluctuating signal as an energy source to drive a reaction away from equilibrium. For example, a protein that has a conformational transition involving charge movement can couple electric energy to drive even a totally non-electrogenic chemical reaction. The interaction is governed solely by intrinsic properties of the enzyme."

This synopsis is an excellent description of a molecular machine – a molecule that can be designed to harvest energy from a specific type of input and use that energy to drive a different process – an output – away from equilibrium thereby doing work and/or storing energy.

A point of controversy has to do with whether a mechanical or a chemical perspective is the most appropriate way to think about molecular machines. The tension between these two points of view is highlighted by the debate[50] between Eric Drexler and Rick Smalley published in Chemical and Engineering News where Smalley points to several insuperable difficulties with Drexler's proposal[51] for a "molecular assembler". This debate echoes more recently in the discussion of

whether binding and reaction of a substrate (fuel) to a molecular machine cause "violent kicks"[52], or whether the overall mechanism involves mechanically equilibrated motions of the individual molecule that can be described as biased diffusion on a sculpted energy landscape[12,16,53]. The divergence between mechanical and chemical perspectives is particularly important in the debate regarding whether these molecular machines work by a power-stroke mechanism[54,55], or whether they operate as Brownian motors by an information or energy ratchet[56,57] mechanism. Recent work has made it abundantly clear that while light powered motors and rotors can, and often do, work by a power-stroke mechanism, ground-state motors, whether externally driven to function as energy ratchets or catalysis driven to function as information ratchets, can only operate as Brownian motors.

An overarching goal is to develop molecular machines that rival biomolecular machines in speed and ability to perform work against an applied load with high thermodynamic efficiency and good reliability. In order to do so it will be necessary to develop a deep understanding of the fundamental theory by which their operation can be described. The perspective offered here is that the physical motions of chemically driven molecular machines as they carry out their function are mechanical equilibrium processes consistent with low Reynold's number motion[58-60] in an environment that is thermodynamically out of chemical equilibrium. The mechanism is best described as diffusion on a sculpted energy landscape. The role of disequilibrium between substrate, S, and product, P, where their chemical potentials are different, $\mu_S \neq \mu_P$, is solely to make sure that it is more likely that substrate rather than product binds when the binding site is unoccupied - i.e., the directed functional motion is due to mass action. Further, there is no loss due to friction, thus explaining how molecular machines can carry out their function with nearly unit efficiency[61].

In this *Scientific Perspective* I present a general approach to theoretical description of molecular machines based on trajectory thermodynamics[40,62] that provides a consistent framework for a theory of molecular motors, rotors, and pumps, as well as for other non-equilibrium chemical phenomena[63] such as driven self-assembly[64,65], molecular adaptation[66-69], and single enzyme chemotaxis[53]. Standard thermodynamic approaches focus on the states of a system, and their free energies. In contrast, trajectory thermodynamics focusses on forward and microscopic reverse trajectories between the states, and on the relations between the probabilities of these trajectories and the energy exchanged with the environment dictated by the principle of microscopic reversibility[70-72] (MR). Using this theoretical backdrop several experimental examples of molecular machines will be discussed, with a focus on the difference between light driven machines, and molecular machines that carry out their function in the ground state.

While the mechanisms of light driven and catalytically driven molecular machines can be superficially similar, deeper analysis shows that the fundamental mechanisms of operation are entirely different. Light driven motors, governed by the Einstein relations[73] for absorption and emission of light, often work by a power-stroke mechanism[19] where the design principle involves using light to raise a system to a high energy state from which it relaxes directionally by a „power-stroke" to a lower energy state. In contrast, catalysis driven motors, governed by microscopic reversibility, function by a Brownian information ratchet mechanisms[45,56,74] where directionality is determined by kinetic asymmetry[48]. External modulation driven motors function by an energy ratchet mechanism[28,29,38] where the input energy derives from the external modulation of a molecular state of the machine, but where the direction is governed by kinetic asymmetry.

2. Theoretical Considerations

Three approaches to power a molecular machine are shown in Fig. 1 for rotaxane molecules (pseudo-rotaxane in Fig. 1a), where the movement of a ring from one place to another is coupled to the chemical state of a dumbbell backbone. The isomerization of the ring can be shifted away from equilibrium by pumping the chemical state of the backbone by light[75] (Fig. 1a), by modulation of the reduction/oxidation (redox) potential[76] (Fig. 1b), or by the catalysis of a chemical reaction[77,] di-isopropyl carbodiimide to di-isopropyl urea (Fig. 1c). In section 2.1 the constraints relevant for light driven motors are discussed, and in section 2.2 the general theory of trajectory thermodynamics[40,78] for non-equilibrium pumping on the ground state by either external modulation of thermodynamic parameters or by catalysis of an exergonic chemical reactions is presented. The somewhat mathematical nature of the discussion is justified by the central role of these quantitative concepts for understanding non-equilibrium chemistry. In section 3 several experimental examples of systems that are pumped away from equilibrium are discussed, and in section 4 the discussion is concluded with speculations on the direction of the field.

At equilibrium the concentration ratio between two isomers of a molecule, A and B, is $\frac{[B]_{eq}}{[A]_{eq}} = K_{AB} = e^{\Delta G_{AB}/RT}$. The basic free energy difference $\Delta G_{AB} = (G_A - G_B)$ is a path independent term - a state function. At equilibrium, kinetics plays no role in

a) Light driven

b) External modulation driven

reduce/oxidize   reduce/oxidize
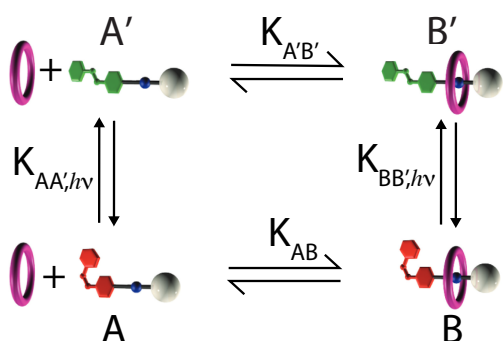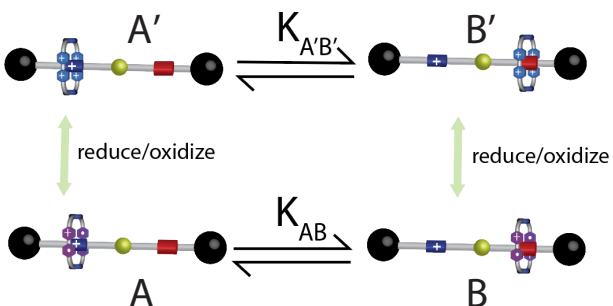
c) Chemical catalysis driven

HOBt   DIC+ HOBt   HOBt   DIC+ HOBt

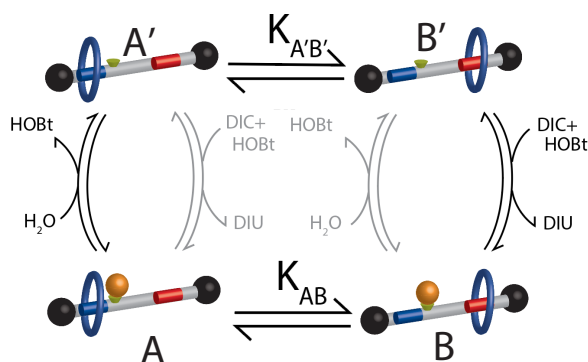H_2O DIU H_2O DIU

Figure 1. Examples of a mechanically bonded two-state rotaxane that can be pumped away from equilibrium such that $\frac{[B]_{ss}}{[A]_{ss}} \neq \frac{[B]_{eq}}{[A]_{eq}}$. a) Pumping by light. b) Pumping by external modulation of some thermodynamic parameter, the reduction/oxidation (redox) potential in this case. c) Pumping by catalysis of diisopropyl carbodiimide (DIC) to diisopropyl urea (DIU), with concomittant protection of the attack site by HOB*t*. The transitions are modelled as dichotomic processes – either the ring changes position, or the backbone dumb-bell or pseudo dumb-bell changes state. The dependence of the position of the ring on the change in state of the backbone, and *vice-versa*, occurs by modification of the rates of the backbone state change depending on the position of the ring, and of the rates for movement of the ring depending on the chemical state of the backbone. The thermodynamic interaction between the HOB*t* and the small blue ring is reciprocal. If the HOB*t* destabilizes the ring on the blue site, occupancy of the blue site decreases the affinity for HOB*t*, and *vice versa*. The kinetic effect, on the other hand, is not, in general reciprocal. Protection by HOB*t* can favor either state A or B, depending on the kinetic asymmetry, but this kinetic preference is manifest only when the catalysed reaction is away from equilibrium.

determining the concentrations of molecular species. Away from equilibrium, however, kinetics becomes critically important, and in calculating the steady state ratio between A and B all paths must be considered and a kinetically weighted ratio of the probabilities of trajectories between paths that lead from A to B vs. paths that lead from B to A must be calculated to determine the steady state ratio between the concentrations of A and B. The direct isomerizations A ⇌ B and A' ⇌ B' are equilibrium

transitions, with equilibrium constants $K_{AB} = \frac{k_{AB}}{k_{BA}} = e^{\Delta G_{AB}/RT}$ and $K_{A'B'} = \frac{k_{A'B'}}{k_{B'A'}} = e^{\Delta G_{A'B'}/RT}$, irrespective of whether the overall system is or is not in thermodynamic equilibrium.

At thermodynamic equilibrium the transitions $A \overset{K_{AA'}}{\rightleftharpoons} A'$ and $B \overset{K_{BB'}}{\rightleftharpoons} B'$ are also governed by equilibrium constants such that $K_{AA'}K_{A'B'} = K_{AB}K_{BB'}$. Away from equilibrium the transitions between A and A', and between B and B', can be pumped by some external energy to drive the process. Let us focus on understanding the constraints of pumped reactions in the context of the transition between A and A',

$$A \underset{k(A' \to A)}{\overset{k(A \to A')}{\rightleftharpoons}} A' \qquad\qquad \text{Scheme 1}$$

If the molecule is optically active, as in the case of the *E* to *Z* transition for the azobenzene substituent shown in Fig. 1 a), light can be used to pump the reaction and maintain a situation in which $\frac{k(A \to A')}{k(A' \to A)} \neq K_{AA'}$ but pumping can also be accomplished in the ground state by external fluctuations of thermodynamic parameters (Fig. 1 b), or by coupling to an exergonic chemical reaction (Fig. 1 c). The constraints on $\frac{k(A \to A')}{k(A' \to A)}$ for these various approaches depend differently on the details of the energy input. Accordingly the design principles for incorporating chemically or thermodynamically pumped reactions in a larger network to create machine-like behavior are very different than the design principles for using light driven reactions.

2.1 *Light driven process*

For the photochemical processes shown in Fig. 1 a), Ragazzon et al. demonstrated[75] experimentally that $\frac{k(A \to A')}{k(A' \to A)} \frac{k(B' \to B)}{k(B \to B')} = K_{AA',h\nu}K_{BB',h\nu}^{-1} \approx 1$. This result is consistent with the Einstein relations for absorption and emission of light where the photophysical properties of A and B are the same. See ref. (79) for a more in-depth treatment of light driven processes. Note that for light driven processes, $K_{AA',h\nu}K_{BB',h\nu}^{-1}$ is not in general proportional to the non-pumped equilibrium constants $K_{AA'}K_{BB'}^{-1}$. When a molecule of A absorbs a photon and is promoted to state A' the energy stored in a single molecule by the light driven process can be used to perform mechanical work in the subsequent thermal relaxation known as a power-stroke. The probability for a clockwise vs. counterclockwise cycle,

$$\frac{k(A \to A')K_{A'B'}k(B' \to B)}{k(A' \to A)K_{AB}k(B \to B')} \approx K_{AB}^{-1}K_{A'B'} \qquad\qquad (1)$$

is proportional to the equilibrium constants for the co-conformational changes. If the direct transition between A and B is very slow, the steady state concentration ratio is $\frac{[B]_{ss}}{[A]_{ss}} \approx K_{A'B'}$ rather than K_{AB} as would be the case at equilibrium. Energy transduction by a light driven motor can often be view in terms of concepts familiar from macroscopic physics where energy is deposited in a machine to place it in a high energy state, and the relaxation from the high energy state – the power stroke – is then used to perform work on the environment. Such ideas have been central in the design of so-called "Feringa" motors[80], where the directionality is an intrinsic property arising from the free-energy differences $\frac{K_{A'B'}}{K_{AB}} = e^{\Delta G_{A'B'}/RT}e^{-\Delta G_{AB}/RT}$.

2.2 *Ground-state driving and kinetic asymmetry*

Designing a system for pumping a reaction in the ground state presents very different challenges than the design of light driven processes. Two examples of ground state pumping are shown in Fig. 1 b and c. In general, input energy, $\delta\varepsilon$, whether from modulation of the redox potential or from catalysis of an exergonic reaction, can shift the reaction in Scheme I to the right or to the left. This results in two distinct reaction channels, each with forward and reverse transitions and rate constants that obey the relations

$$\frac{k_{A \to A'}^{(1)}}{k_{A' \to A}^{(1)}} = K_{AA'} \, e^{\delta\varepsilon/RT} \qquad\qquad (2a)$$

$$\frac{k_{A \to A'}^{(2)}}{k_{A' \to A}^{(2)}} = K_{AA'} \, e^{-\delta\varepsilon/RT} \qquad\qquad (2b)$$

respectively. These relations represent the constraints of MR on the ratios between rate constants for forward and microscopic reverse transitions for any single channel of reaction. Eqs. (2a) and (2b) form the crux of understanding the operation of ground state molecular machines. If reaction channel 1 is "fast" in comparison to channel 2 the input energy shifts the reaction Scheme 1 to the

right in favor of A'. if reaction channel 2 is fast in comparison to channel 1 the input energy shifts the reaction Scheme 1 to the left in favor of A. The directionality is controlled solely by the kinetics, i.e., by which reaction channel is faster than the other.

Some authors describe relations such as Eqs. (2a) and (2b) as "local" or "generalized" detailed balance[81]. These terms, when used as a synonym for MR, are not incorrect, but confusion can result if it is not made clear that such simple exponential relations hold only for the forward and microscopic reverse rate constants for an individual reaction channel, and NOT for the net forward and backward rate constants between two states of a molecular machine. The MR condition for the ratio of forward and reverse rate constants for any channel is sufficient to guarantee thermodynamic consistency of a model. However, in order to preserve the symmetry of the effect of input energy – a scalar quantity – on the system both reaction channels must be taken into consideration. All energy-pumped ground state transitions must have at least two reaction channels since *a priori* the input of energy can shift the reaction either to the left or to the right. Developing a theory by ignoring one of the reaction channels, e.g. Eq (2b), to focus on the other reaction channel, e.g. Eq (2a), falls into the logical fallacy derided by Koenig, Horne, and Mohilner[82] as "coupling by the stroke of a pen" and obscures the key design principle for molecular motors known as kinetic asymmetry. The correct expression for the ratio of forward to backward rate coefficients in Scheme 1 is the ratio of sums $\frac{k(A \to A')}{k(A' \to A)} = \frac{\left(k_{A \to A'}^{(1)} + k_{A \to A'}^{(2)}\right)}{\left(k_{A' \to A}^{(1)} + k_{A' \to A}^{(2)}\right)}$ and

$$\frac{k(A \to A')}{k(A' \to A)} = K_{AA'} \underbrace{\left[\frac{k_{A' \to A}^{(1)} e^{+\delta\varepsilon/RT} + k_{A' \to A}^{(2)} e^{-\delta\varepsilon/RT}}{k_{A' \to A}^{(1)} + k_{A' \to A}^{(2)}}\right]}_{\langle e^{\mathcal{W}_{AA'}/RT} \rangle} \qquad (3)$$

The expression Eq. (3), $\frac{k(A \to A')}{k(A' \to A)} = K_{AA'} \langle e^{\mathcal{W}_{AA'}/RT} \rangle$, is an extension of the concept of an equilibrium constant to a possibly non-equilibrium steady-state. The term $\langle e^{\mathcal{W}_{AA'}/RT} \rangle$ is the exponential of the path dependent energy exchanged with the environment kinetically weighted and averaged over all reaction channels. While here we focus on the case of two reaction channels, the expression $\frac{k(A \to A')}{k(A' \to A)} = K_{AA'} \frac{\sum_{i=0}^{n} k_{A \to A}^{(i)} e^{\mathcal{W}_{AA'}^{(i)}/RT}}{\sum_{i=0}^{n} k_{A' \to A}^{(i)}} = K_{AA'} \langle e^{\mathcal{W}_{AA'}/RT} \rangle$ follows from MR and holds in general for the ratio of net rate constants.

Obviously the ratio of rate constants calculated from Eq. (3) is equal the equilibrium constant if $\delta\varepsilon = 0$ irrespective of the relative rates of channels (1) and (2) since there is no dissipation. This result was termed[70] by Lewis "A New Principle of Equilibrium". If, instead, we have $k_{A' \to A}^{(1)} e^{+\delta\varepsilon/RT} + k_{A' \to A}^{(2)} e^{-\delta\varepsilon/RT} = k_{A' \to A}^{(1)} + k_{A' \to A}^{(2)}$, which holds when $\frac{k_{A' \to A}^{(1)}}{k_{A' \to A}^{(2)}} = e^{-\delta\varepsilon/RT}$, the ratio $\frac{k(A \to A')}{k(A' \to A)} = K_{AA'}$ is the same as at equilibrium despite continual dissipation. This condition is known as kinetic symmetry, the breaking of which – kinetic asymmetry – "is a key factor determining whether an enzyme can use energy driven fluctuation for doing useful output work"[48]. For catalysis driven pumping $\frac{k_{A' \to A}^{(1)}}{k_{A' \to A}^{(2)}}$ is related to the catalytic bias[83,84] so we term the ratio $\frac{k_{A' \to A}^{(1)}}{k_{A' \to A}^{(2)}}$ the kinetic bias. An expression analogous to Eq. (3) holds for the ratio $\frac{k(B \to B')}{k(B' \to B)}$. When multiplied together we find the ratio of the probabilities of a clockwise and counterclockwise cycle, known as the directionality r_0, or in some papers as the ratcheting constant K_r, to be

$$\frac{k(A \to A') k(B' \to B)}{k(A' \to A) k(B \to B')} \frac{K_{A'B'}}{K_{AB}} = \underbrace{\left[\frac{N + \left(e^{\ln q} e^{2\delta\varepsilon/RT} + 1\right)}{N + \left(e^{\ln q} + e^{2\delta\varepsilon/RT}\right)}\right]}_{r_0} \underbrace{\frac{K_{AA'}}{K_{BB'}} \frac{K_{A'B'}}{K_{AB}}}_{1} \qquad (4)$$
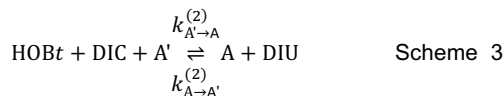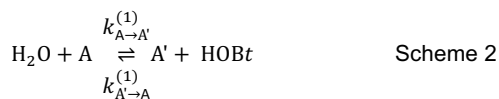
where the term $q = e^{\ln q} = \frac{k_{A' \to A}^{(1)} k_{B' \to B}^{(2)}}{k_{A' \to A}^{(2)} k_{B' \to B}^{(1)}}$ is the overall kinetic bias. This term is written as the exponential of the logarithm to emphasize the fact that the kinetic factor, $\ln q$, and the energetic factor $2\delta\varepsilon/RT$ play equal roles in determining the directionality. The kinetic bias is a property of the molecular machine, and hence the focus of any attempt to design a molecular machine. Note that $\ln q$ depends on the differences of activation energies but does not depend on the free-energies of the states themselves. The term common in the numerator and denominator $N = \left(\frac{k_{A \to A}^{(1)}}{k_{A \to A}^{(2)}} e^{2\delta\varepsilon/RT} + \frac{k_{B' \to B}^{(2)}}{k_{B' \to B}^{(1)}}\right)$ parametrizes the uncoupling. If N is very large, the energy input $2\delta\varepsilon$ is only weakly coupled to driving the molecular machine and leads to only a small directionality. In contrast, the situation $N \to 0$ is known as the complete coupling regime.

If the direct transition between A and B is very slow, the steady state concentration ratio is $\frac{[B]_{ss}}{[A]_{ss}} \approx K_{AB} r_0$. In contrast to the light driven case, here we find that the steady state ratio is equal the equilibrium constant K_{AB} modified by the factor r_0 where $r_0 > 1$ if $(\ln q \times \delta\varepsilon) > 0$ is and $r_0 < 1$ if $(\ln q \times \delta\varepsilon) < 0$. Note that r_0 does not depend on the free energies of any of the states. In the completely coupled regime ($N \to 0$) we find two salient limits from Eq. (3). When $\ln q \to \pm\infty$, $r_0 \to e^{\pm 2\delta\varepsilon/RT}$, and when

$\delta\varepsilon \to \pm\infty$, $r_0 \to e^{\pm \ln q}$. We can see how this works out in a more chemically familiar way in terms of the catalytic process shown in Fig. 1c). The two reactions between A and A' are

$$\text{H}_2\text{O} + \text{A} \underset{k^{(1)}_{\text{A'}\to\text{A}}}{\overset{k^{(1)}_{\text{A}\to\text{A'}}}{\rightleftharpoons}} \text{A'} + \text{HOB}t \qquad\qquad \text{Scheme 2}$$

$$\text{HOB}t + \text{DIC} + \text{A'} \underset{k^{(2)}_{\text{A}\to\text{A'}}}{\overset{k^{(2)}_{\text{A'}\to\text{A}}}{\rightleftharpoons}} \text{A} + \text{DIU} \qquad\qquad \text{Scheme 3}$$

The rate constants are constrained by the MR relations

$$\frac{k^{(1)}_{\text{A}\to\text{A'}}[\text{H}_2\text{O}]}{k^{(1)}_{\text{A'}\to\text{A}}[\text{HOB}t]} = \text{K}_{\text{AA'}}\, e^{\mu_{\text{H}_2\text{O}}} \tag{5a}$$

$$\frac{k^{(2)}_{\text{A}\to\text{A'}}[\text{UIC}]}{k^{(2)}_{\text{A'}\to\text{A}}[\text{HOB}t][\text{DIC}]} = \text{K}_{\text{AA'}}\, e^{(\mu_{\text{DIU}}-\mu_{\text{DIC}})} \tag{5b}$$

where $\text{K}_{\text{AA'}} = e^{[(G_\text{A}-G_{\text{A'}})-\mu_{\text{HOB}t}]}$ is the equilibrium constant for attachment of the blocking group HOBt. Using Eqs. (5a) and (5b) we find the ratio of net rate constants

$$\frac{k(\text{A}\to\text{A'})}{k(\text{A'}\to\text{A})} = \text{K}_{\text{AA'}} \underbrace{\left[\frac{\frac{k^{(1)}_{\text{A'}\to\text{A}}}{k^{(2)}_{\text{A'}\to\text{A}}[\text{DIC}]}e^{\mu_{\text{H}_2\text{O}}/RT}+e^{\mu_{\text{DIU}}-\mu_{\text{DIC}}/RT}}{\frac{k^{(1)}_{\text{A'}\to\text{A}}}{k^{(2)}_{\text{A'}\to\text{A}}[\text{DIC}]}+1} \right]}_{\mathcal{A}_\text{A}} \tag{6}$$

The factor $\mathcal{A}_\text{A}$ is the kinetic asymmetry parameter[45]. A similar equation holds for the transitions between B and B'. By multiplication we get

$$\frac{k(\text{A}\to\text{A'})k(\text{B'}\to\text{B})}{k(\text{A'}\to\text{A})k(\text{B}\to\text{B'})}\frac{\text{K}_{\text{A'B'}}}{\text{K}_{\text{AB}}} = \mathcal{A}_\text{A}\mathcal{A}_\text{B}^{-1} = \underbrace{\left[\frac{N+\left(e^{\ln q}e^{\Delta\mu/RT}+1\right)}{N+\left(e^{\ln q}+e^{\Delta\mu/RT}\right)} \right]}_{r_0} \tag{7}$$

where $\Delta\mu = \mu_{\text{H}_2\text{O}} + \mu_{\text{DIC}} - \mu_{\text{DIU}}$, $q = e^{\ln q} = \frac{k^{(1)}_{\text{A'}\to\text{A}}\tilde{k}^{(2)}_{\text{B'}\to\text{B}}}{\tilde{k}^{(2)}_{\text{A'}\to\text{A}}k^{(1)}_{\text{B'}\to\text{B}}}$, $N = \left(\frac{k^{(1)}_{\text{A'}\to\text{A}}}{\tilde{k}^{(2)}_{\text{A'}\to\text{A}}}e^{\Delta\mu/RT} + \frac{\tilde{k}^{(2)}_{\text{B'}\to\text{B}}}{k^{(1)}_{\text{B'}\to\text{B}}} \right)$, and the tilde indicates a pseudo first-order rate coefficient into which the concentration [DIC] has been subsumed. The latter identity follows from the thermodynamic relation $\text{K}_{\text{AA'}}\text{K}_{\text{A'B'}}\text{K}_{\text{B'B}}\text{K}_{\text{BA}} = 1$. 3The rate constants $k^{(1)}_{\text{A'}\to\text{A}}$ and $k^{(1)}_{\text{B'}\to\text{B}}$ in the expression for q are for processes that seldom if ever occur and are hence not directly accessible by experiment. Their values however can be determined experimentally by use of MR, allowing the kinetic bias factor to be written in terms of equilibrium constants and rate constants for esperimentally accessible processes

$$q = \frac{k^{(1)}_{\text{A}\to\text{A'}}\tilde{k}^{(2)}_{\text{B'}\to\text{B}}}{\tilde{k}^{(2)}_{\text{A'}\to\text{A}}k^{(1)}_{\text{B}\to\text{B'}}}\text{K}_{\text{AB}}^{-1}\text{K}_{\text{A'B'}} \tag{8}$$

At first glance it might seem that Eq. (8) argues for the importance of the power stroke – the free-energy differences $\Delta G_{\text{A'B'}} = RT \ln \text{K}_{\text{A'B'}}$ and $\Delta G_{\text{BA}} = RT \ln \text{K}_{\text{AB}}^{-1}$ – as a determinant of the directionality as is the case for light driven processes highlighted in Eq. (1). This conclusion however is illusory. The combination of rate constants $\frac{k^{(1)}_{\text{A}\to\text{A'}}\tilde{k}^{(2)}_{\text{B'}\to\text{B}}}{\tilde{k}^{(2)}_{\text{A'}\to\text{A}}k^{(1)}_{\text{B}\to\text{B'}}}$ in Eq. (8) is proportional to $\text{K}_{\text{AA'}}\text{K}_{\text{BB'}}^{-1} = \text{K}_{\text{AB}}\text{K}_{\text{A'B'}}^{-1}$ and hence the equilibrium constants cancel out in the product shown, leaving behind only terms involving transition state energies. There can be no controversy or ambiguity about the mathematically deductive result that the directionality depends only on the difference in transition state energies and does not depend on the basic free-energies of the states. It may, however, be that a chemical modification to a molecular machine that is designed to change, e.g., the free-energy of one of the states and hence the equilibrium constant associated with a power-stroke also results in a change to a transition state energy and hence to q and the directionality. This point was addressed[41] by Amano et al. from the experimental perspective.

2.3. *The non-equilibrium pumping equality*

Microscopic reversibility holds not only for elementary reaction channels, but also for forward and reverse trajectories, $\mathcal{S}^i_{\text{AB}}$ and $\mathcal{S}^i_{\text{BA}}$, in general, according to the relation

$$\frac{\pi\left(A\xrightarrow{s^i_{AB}}B\right)}{\pi\left(B\xrightarrow{s^i_{BA}}A\right)} = K_{AB}e^{\mathcal{W}(s^i_{AB})/RT} \tag{9}$$

Where $\mathcal{W}(s^i_{AB})$ is the excess energy exchanged with the environment in the trajectory s^i_{AB}. It is important to note that describing this excess energy as "dissipation" or "entropy production" is a conceptual error and is seriously misleading. Unlike dissipation or entropy production, $\mathcal{W}(s^i_{AB})$ can be either positive or negative, with the sign determining whether A or B is favored relative to equilibrium in the trajectory i. Some trajectories can have positive $\mathcal{W}(s^i_{AB})$ and others negative $\mathcal{W}(s^i_{AB})$ and it is the kinetic weighting that ultimately determines whether A or B is favored under a specific type of non-equilibrium constraint. The steady-state (ss) between A and B occurs when the probability for a trajectory from A to B is equal the probability for a trajectory from B to A,

$$[A]_{ss}\sum_i\pi\left(A\xrightarrow{s^i_{AB}}B\right) = [B]_{ss}\sum_i\pi\left(B\xrightarrow{s^i_{BA}}A\right) \tag{10}$$

Eq. (10), by use of Eq. (9), is[49]

$$\frac{[B]_{ss}}{[A]_{ss}} = K_{AB}\underbrace{\frac{\sum_i\pi\left(B\xrightarrow{s^i_{BA}}A\right)e^{\mathcal{W}(s^i_{AB})/RT}}{\sum_i\pi\left(B\xrightarrow{s^i_{BA}}A\right)}}_{=\langle e^{\mathcal{W}(s^i_{AB})/RT}\rangle} \tag{11a}$$
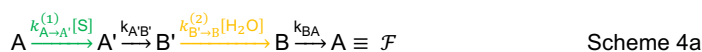
Which can be rearranged to the relation

$$\Delta\mu_{BA} = RT\ln\langle e^{\mathcal{W}(s^i_{AB})/RT}\rangle \tag{11b}$$

known as the non-equilibrium pumping equality. This equality between the steady state chemical potential difference and the exponential of the excess work exchanged with the environment kinetically weighted and averaged over all trajectories (not just elementary reactions) between A and B. Eqs. (11a) and (11b) are principle results of trajectory thermodynamics[40,78] and provides a conceptual basis for understanding how non-equilibrium conditions lead to mainainance of a chemical potential difference between two states. The relationship between the equilibrium constants is such that $K_{AA'}K_{A'B'}K_{B'B} = K_{AB}$. Such a simple equality is NOT TRUE, however, for the products of the exponentials of the energy exchange averaged over all reaction channels. In a setup where the direct transition between the two states A and B is slow, energy input can lead to a steady state non-equilibrium concentration difference. Binks et al. have recently tested[77] the prediction that for very slow direct transfer between A and B the steady state ratio is linearly proportional to the equilibrium ratio with, $\frac{[B]_{ss}}{[A]_{ss}} \approx \frac{[B]_{eq}}{[A]_{eq}}r_0$, finding that indeed this relationship does hold, with a value $r_0 = 20.6$ for the system[shown in Fig. 1 c) powered by conversion of DIC to UIC to provide the energy for non-equilibrium installation and hydrolysis of the HOBt protecting group.

3. Experimental examples

3.1 *Cyclic reactions, trajectory thermodynamics, and energy landscapes*

The catalytically driven molecular machine of Borsley, Keidt, Leigh, and Roberts rotates directionally about a single bond[22], with mechanical motion interleaved[85] with chemistry. The molecular mechanism is shown as a cyclic process in Fig. 2a). In the experiment of Borsley et al. substrate S was DIC and product P was UIC. There are two cycles in which S is converted to P, one in which the rotor undergoes clockwise rotation when viewed from the perspective in which the pyrole rotor is closer than the phenol stator to the observer

$$A\xrightarrow{k^{(1)}_{A\to A'}[S]}A'\xrightarrow{k_{A'B'}}B'\xrightarrow{k^{(2)}_{B'\to B}[H_2O]}B\xrightarrow{k_{BA}}A \equiv \mathcal{F} \qquad\qquad \text{Scheme 4a}$$

And another in which the rotor undergoes counter-clockwise rotation from that same perspective

$$A\xrightarrow{k_{AB}}B\xrightarrow{k^{(1)}_{B\to B'}[S]}B'\xrightarrow{k_{B'A'}}A'\xrightarrow{k^{(2)}_{A\to A'}[H_2O]}A \equiv \mathcal{B} \qquad\qquad \text{Scheme 4b}$$
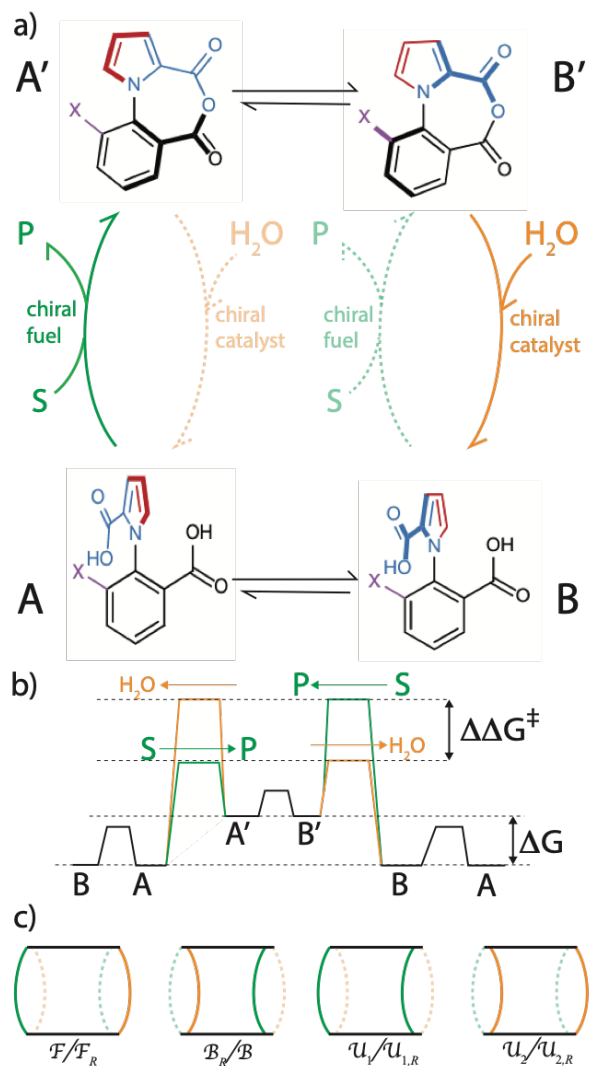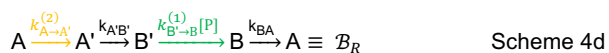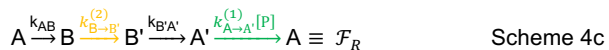
Figure 2. Illustration of a chemically driven molecular machine that directionally rotates about a single bond. a) 4-state kinetic cycle for a catalysis driven molecular rotor. b) energy landscapes for molecular rotor in 2 a). The fuel is chiral which allows experimental selection of the barrier for conversion of S→P by state A to be lower than conversion of S→P by state B. Further, the catalyst that facilitates hydrolysis is also chiral, and the hydrolysis is faster in the B' and B states. The fact that both barriers can be influenced by experimental choices leads to the description as a "doubly gated chemically driven information ratchet". c) skeleton mechanisms for the four state cycles that contribute to the turning of the rotor.

The probabilities for these cycles, $\pi_{\mathcal{F}}$ and $\pi_{\mathcal{B}}$, are proportional to the products of the rate constants, and the proportionality constant in the denominator is the same for both cycles. Thus, the ratio $\frac{\pi_{\mathcal{F}}}{\pi_{\mathcal{B}}} = q$ is
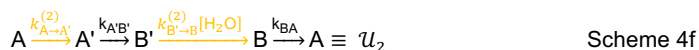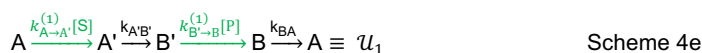
proportional to the net kinetic bias given by Eq. (8). Each of these two cycles have microscopic reverse cycles,

$$A \xrightarrow{k_{AB}} B \xrightarrow{k_{B\rightarrow B'}^{(2)}} B' \xrightarrow{k_{B'A'}} A' \xrightarrow{k_{A\rightarrow A'}^{(1)}[P]} A \equiv \mathcal{F}_R \qquad \text{Scheme 4c}$$

$$A \xrightarrow{k_{A\rightarrow A'}^{(2)}} A' \xrightarrow{k_{A'B'}} B' \xrightarrow{k_{B'\rightarrow B}^{(1)}[P]} B \xrightarrow{k_{BA}} A \equiv \mathcal{B}_R \qquad \text{Scheme 4d}$$

The cycles $\mathcal{F}_R$ and $\mathcal{B}_R$ involve conversion of P to S, and hence are unlikely if $\mu_S \gg \mu_P$. Nevertheless, it is essential to include these cycles to obtain a thermodynamically consistent theoretical description of a molecular machine, where the ratios $\frac{\pi_{\mathcal{F}}}{\pi_{\mathcal{F}_R}} = \frac{\pi_{\mathcal{B}}}{\pi_{\mathcal{B}_R}} = e^{\Delta\mu_{SP}/RT}$ are essential for simplification of the expression of the directionality.

The Schemes 4a-4d represent all coupled cycles and hence in the fully coupled limit the ratio of the probability for a clockwise vs. counterclockwise cycle, i.e. the directionality in the completely coupled limit is found to be $\frac{\pi_{\mathcal{F}}+\pi_{B_R}}{\pi_B+\pi_{\mathcal{F}_R}} = \frac{e^{\ln q}e^{\Delta\mu_{SP}/RT}+1}{e^{\Delta\mu_{SP}/RT}+e^{\ln q}}$. There are also uncoupled cycles

$$A \xrightarrow{k_{A\rightarrow A'}^{(1)}[S]} A' \xrightarrow{k_{A'B'}} B' \xrightarrow{k_{B'\rightarrow B}^{(1)}[P]} B \xrightarrow{k_{BA}} A \equiv \mathcal{U}_1 \qquad\qquad \text{Scheme 4e}$$

$$A \xrightarrow{k_{A\rightarrow A'}^{(2)}} A' \xrightarrow{k_{A'B'}} B' \xrightarrow{k_{B'\rightarrow B}^{(2)}[H_2O]} B \xrightarrow{k_{BA}} A \equiv \mathcal{U}_2 \qquad\qquad \text{Scheme 4f}$$

and their microscopic reverses,

$$A \xrightarrow{k_{AB}} B \xrightarrow{k_{B\rightarrow B'}^{(1)}[S]} B' \xrightarrow{k_{B'A'}} A' \xrightarrow{k_{A'\rightarrow A}^{(1)}[P]} A \equiv \mathcal{U}_{1,R} \qquad\qquad \text{Scheme 4g}$$

$$A \xrightarrow{k_{AB}} B \xrightarrow{k_{B\rightarrow B'}^{(2)}} B' \xrightarrow{k_{B'A'}} A' \xrightarrow{k_{A'\rightarrow A}^{(2)}[H_2O]} A \equiv \mathcal{U}_{2,R} \qquad\qquad \text{Scheme 4h}$$
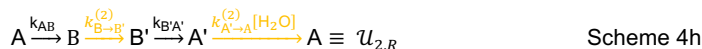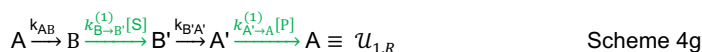
The cycles Scheme 4a-h are shown in "skeleton" form[48] in Fig. 2 c), where the bold lines indicate which transitions are considered in the cycle. The overall directionality is calculated as the ratio of the sum of the clockwise probabilities to the sum of the counterclockwise probabilities, which when multiplied out reduces to the ratcheting equation[57]

$$r_0 = \frac{\pi_{\mathcal{F}}+\pi_{B_R}+\pi_{\mathcal{U}_1}+\pi_{\mathcal{U}_2}}{\pi_B+\pi_{\mathcal{F}_R}+\pi_{\mathcal{U}_{1,R}}+\pi_{\mathcal{U}_{2,R}}} = \frac{N+(e^{\ln q}e^{\Delta\mu_{SP}/RT}+1)}{N+(e^{\Delta\mu_{SP}/RT}+e^{\ln q})} \qquad (12)$$
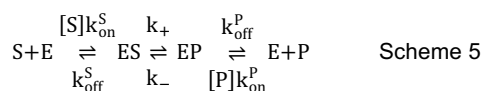
Where each cycle probability (π_i) in Eq. (12) is the product of the rate constants in the corresponding cycle.

The key to understanding the directionality of a cyclic process lies in a recognition of the importance of kinetic asymmetry for determining the ratio of forward to backward rate constants given in Eqs. (3) and (6). When we use these expressions we find that the ratio of the probability for a clockwise cycle to a counterclockwise cycle, given as the ratio of the products of the clockwise and counterclockwise rate constants, is $\frac{k(A\rightarrow A')k(B'\rightarrow B)}{k(A'\rightarrow A)k(B\rightarrow B')}K_{AB}^{-1}K_{A'B'} = r_0$, which is independent of the free energies of the states. In general, any cycle where the transitions, pumped or not, involve ground state processes, the equilibrium constants cancel, leaving behind only the free-energy input and the kinetic bias as the determinant of directionality. This independence of the cycle direction on the equilibrium constants can be lifted in the presence of spatial gradients[53,86]. The cycling through the states in a particular order can describe, among many other possibilities, rotation of a rotor, directional walking of a molecular walker of a track. The directionality is attained at the expense of consuming substrate, where the available energy per stoichimetric conversion of substrate to waste product is denoted $\Delta\mu_{SP}$, or harvested from external perturbations, where the available energy per stoichimetric conversion of substrate to waste product is denoted $2\delta\varepsilon$. It is essential to recognize that there is a backward process where the motor or rotor moves backward while still converting S to P. This behavior was predicted[87] for a Brownian motor in 1996, but wasn't observed experimentally[88] until 2005 when Carter and Cross, using single molecule techniques, applied a strong enough external force to cause kinesin to move backward. The velocity of backward motion was increased by the addition of ATP as predicted by trajectory thermodynamics[87] rather than impeded as predicted by a tight-coupling model[89].
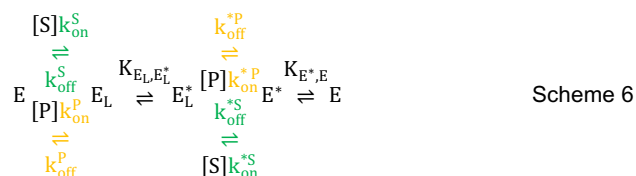
The directionality, r_0 (or equivalently the ratchetting constant K_r), does not depend on the free-energies of the states – thermodynamics - but only on the transition state energies – kinetics. The four-state cycle Fig. 2a) is the same as that studied in Astumian et al.[48] to establish kinetic asymmetry as a key property necessary for enzymes to harness energy from external modulation of thermodynamic parameters. Penocchio and Ragazzon[90] have shown how the two energy landscapes shown in Fig. 2 b) can be converted into a single kinetic barrier diagram[90-92] with a net slope indicative of the directionality. Here we have focused on how kinetic asymmetry allows catalysis to drive the reaction to cycle preferentially in a particular order A→A'→B'→B→A.

3.2 *Common misconceptions about catalytic cycles*

It is important to confront head-on the fact that a very common way to describe an enzyme that catalyzes the reaction $S \overset{K_{eq}}{\rightleftharpoons} P$,

$$S+E \underset{k_{off}^S}{\overset{[S]k_{on}^S}{\rightleftharpoons}} ES \underset{k_-}{\overset{k_+}{\rightleftharpoons}} EP \underset{[P]k_{on}^P}{\overset{k_{off}^P}{\rightleftharpoons}} E+P \qquad \text{Scheme 5}$$

is seriously misleading. Scheme 5 can be cast in the form of a triangle reaction[93] and is used to claim that the directionality can be understood in terms of a thermodynamic cycle driving force or affinity, in this case $X = RT \ln\left(\frac{[S]}{[P]} K_{eq}\right) = \Delta\mu_{SP}$. This assertion is not correct – the direction of cycling is determined by kinetic asymmetry, and the effective driving force is a kinetically weighted average of $e^{\Delta\mu_{SP}/RT}$ and $e^{-\Delta\mu_{SP}/RT}$. The concept of a cycle affinity pertains only in the limit $\ln q \to \pm\infty$. In general the kinetic asymmetry determines the directionality of the cycle[16]. Scheme 5a, in distinguishing ES and EP, implicitly assumes that the chemical potentials of bound S and P can be independently defined. Publication[94] of this incorrect idea led to a very fruitful literature exchange between Terrell Hill[95-97] and Charles Tanford[98-100]. Tanford argued that the chemical potential of a reactant such as ATP can be followed through a catalytic cycle, and that the specific step in which ATP is hydrolyzed *in situ* to ADP + Pi, and the subsequent conformation change of the enzyme, are key to understanding free-energy transduction by biomolecular machines. Hill demonstrated that different forms of bound ligands with the same stoichiometry do not have independently definable chemical potentials[97]. This distinction was incorporated in the models discussed recently by Feng et al.[12] who discussed a more consistent way to write Scheme 5,

$$E \; \overset{[S]k_{on}^{S}}{\underset{k_{off}^{S}}{\rightleftharpoons}} \; \underset{[P]k_{on}^{P}}{\overset{}{\rightleftharpoons}} \; E_{L} \overset{K_{E_L,E_L^*}}{\rightleftharpoons} E_L^* \; \underset{[P]k_{on}^{*P}}{\overset{k_{off}^{*P}}{\rightleftharpoons}} \; \underset{[S]k_{on}^{*S}}{\overset{k_{off}^{*S}}{\rightleftharpoons}} \; E^* \overset{K_{E^*,E}}{\rightleftharpoons} E \qquad\qquad \text{Scheme 6}$$

Where E and E^* are different conformations of the unbound state of the catalyst, and E_L and E_L^* are different conformations of the bound state of the catalyst, and can be described by the four-state diagram[48] in Fig. 2c). Although unwieldy, this way of writing an enzyme reaction captures the necessity of having two channels for reaction between any "free" state and any "bound" state of a catalyst. It makes no sense to imagine one state of a catalyst that can bind/release substrate but not product, and another conformational state that can bind/release product but not substrate. Such a situation is not thermodynamically consistent since the ratios of the rate constants are constrained by microscopic reversibility[56,87]

$$\frac{k_{on}^{S}k_{off}^{P}}{k_{off}^{S}k_{on}^{P}} = \frac{k_{on}^{*S}k_{off}^{*P}}{k_{off}^{*S}k_{on}^{*P}} = \frac{k_{on}^{S}k_{off}^{*P}}{k_{off}^{S}k_{on}^{*P}}K_{E_L,E_L^*}K_{E^*,E} = \frac{k_{on}^{*S}k_{off}^{P}}{k_{off}^{*S}k_{on}^{P}}K_{E_L,E_L^*}^{-1}K_{E^*,E}^{-1} = K_{eq} \quad (13)$$

The overall kinetic bias, $q = \frac{k_{off}^{S}k_{off}^{*P}}{k_{off}^{P}k_{off}^{*S}}$ is not constrained by the values of $K_{E_L,E_L^*}, K_{E^*,E}, K_{eq}, [S]$, or $[P]$. With $\Delta\mu_{SP} > 0$, the enzyme cycles through the states predominately in the order $E \to E_L \to E_L^* \to E^* \to E$ if $q > 1$, and in the order $E \to E^* \to E_L^* \to E_L \to E$ if $q < 1$. Kinetic asymmetry is the key determinant by which directional cycling is engendered, and a key factor allowing an enzyme to use non-equilibrium fluctuations to perform work[48]. The directionality of the enzyme model Scheme 6 is the ratio of the net rate constants $r_0 = \frac{([S]k_{on}^{S}+[P]k_{on}^{P})(k_{off}^{*S}+k_{off}^{*P})}{([S]k_{on}^{*S}+[P]k_{on}^{*P})(k_{off}^{S}+k_{off}^{P})}K_{E_L,E_L^*}K_{E^*,E}$ \qquad (14)

Which can be cast into the form of Eq. (7). Use of the identity $K_{E,E_L}K_{E_L^*,E^*} = K_{E_L,E_L^*}^{-1}K_{E^*,E}^{-1}$ makes it clear that the power-stroke plays no role whatsoever in determining r_0. There has been a resurgence of interest in examining the constraints on kinetic cycles[101] and how these cycles can be optimized for free-energy transduction[102].

Hill and his colleague Evan Eisenberg went on to recognize that free-energy transduction cannot be localized to some crucial steps in an enzyme cycle but must be viewed in the context of the whole cycle, observing that "though the controversy is only conceptual, it is not insignificant, because much mental and some experimental effort is being expended in trying to locate the crucial step or the 'energized state'[2] in transduction cycles". The tendency to attempt to localize energy transduction in ATP driven machines remains[55] to this day, where the 'critical step' is illustrated graphically with a lightning bolt and the words "fuel event". This way of thinking appears to derive from concepts of light driven processes. Hill and Eisenberg recognized[97] a crucial difference between ATP driven and light driven processes. In the latter, a single step – the absorption of a photon – can in fact be identified as the critical step of energy transduction and the subsequent downhill relaxation is appropriately described as a power-stroke. The effect of ATP hydrolysis, in contrast, must be viewed in the context of the entire catalytic cycle, and the only correct description of the role of ATP is in terms of mass action. At the time Hill and Eisenberg's paper[97] was written the discussion was indeed only conceptual, but with the great advances in single molecule experiments on biomolecular machines, and the design and synthesis of artificial molecular machines, the controversy between Hill and Tanford has become experimentally testable[16].

a) Light driven rotor
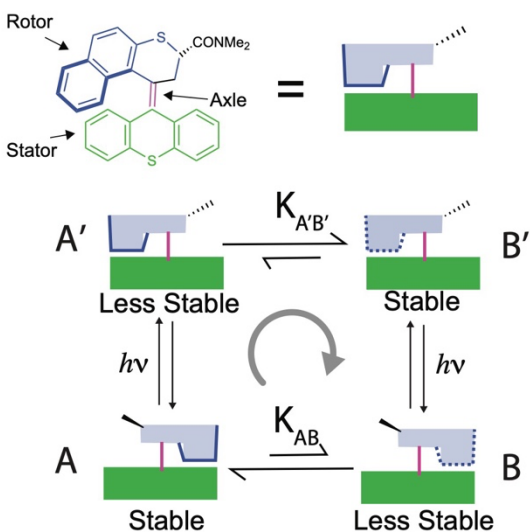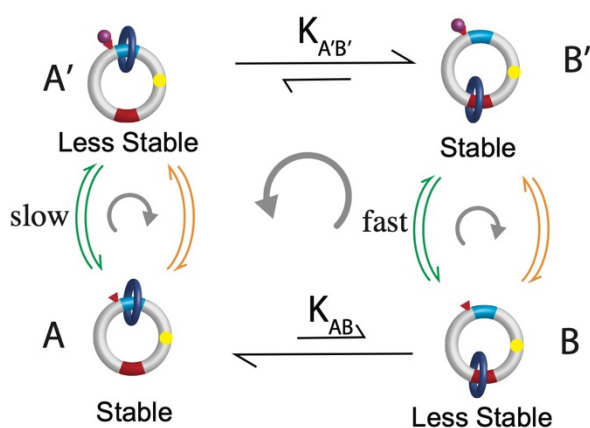

b) catalysis driven rotor

Figure 3 Comparison between a light driven motor and a catalysis driven motor. In a), absorption of a photon when in state A or in B' promotes the molecule to a high enrgy state A' or B, respectively, from which the system undergoes directional relaxation. This relaxation process is known as a power-stroke. b). It might seem that a catalysis driven process could be designed according to a similar principle in which chemical energy is used to increase the energy of the system from stable state A to high energy state A', with subsequent directional relaxation to B' giving rise to cycling A→A'→B'→B→A. In fact, catalysis drives the cycle A→B→B'→A'→A by kinetic asymmetry – the catalytic addition of HOB*t* to B is faster than to A because of steric effects.

3.3 *Light vs. Catalysis driven cycles*

Consider the light driven rotor shown in Fig. 3a) in comparison to the catalysis driven rotor in Fig. 3b). In 3a) photon absorption provides a mechanism by which a molecule in stable state A is lifted to the higher energy unstable state A'. Relaxation from this unstable state by ring inversion to the stable state B' is spontaneous. Absorption of a photon then lifts the molecule to the unstable state B from which relaxation to the stable state A is spontaneous, and by continual absorption of a photon followed by thermal relaxation the molecule undergoes continual rotation.

Now consider a catalysis driven rotor inspired by that presented by Wilson et al.[21] (Fig. 3 b), with energy provided by the catalytic placement of a fluoro-methoxy carbonyl chloride (Fmoc) protecting group, which spontaneously cleaves to form dibenzo-fulvene (DBFV). Fmoc→DBFV releases approximately as much energy as hydrolysis of ATP.

At first glance it might seem that this rotor works similarly to the light driven rotor, with chemical energy being used to lift a molecule in state A to the unstable state A' by binding the bulky protecting group to destabilize the navy ring on the otherwise

very stable aqua recognition (binding) site followed by thermal relaxation of the ring over the yellow fiducial group to the now relatively more stable red recognition site (state B'). Upon cleavage of the Fmoc to form DBFV the navy ring returns to the stable aqua recognition site, with the net effect being that the navy ring undergoes more cycles in the order aqua→yellow→red→aqua than in the order aqua→red→yellow→aqua. This is, in fact, NOT the case. The cycle aqua→red→yellow→aqua is MORE LIKELY than the cycle aqua→yellow→red→aqua. In order to understand this result I have stripped the inessential elements leaving behind only two – the thermodynamic influence of the relative stabilities of the states, and the kinetic influence due to the steric hindrance to the catalytic addition of the protecting group when the small ring is near the site of attack. The thermodynamic effect cancels out in the product of the ratios of the net rate constants. The kinetic effect does not. Because the addition of Fmoc is faster when the navy ring is on the red site, the cycling through states occurs preferentially in the order A→B→B'→A'→A, and the transition B'→A' can only occur over the yellow fiducial group so the motion of the small ring aqua→red→yellow→aqua is the predominate direction of rotation. The directionality does not depend in any way on the equilibrium constants K_{AB} or $K_{A'B'}$.

We can also understand how kinetic asymmetry explains directionality of molecular machines in the context of more complicated systems in the context of two-dimensional energy diagrams inspired by work on the F1 ATP synthase.

2.4 *F1 ATP synthase and a synthetic electric motor*

Computational studies on the FoF1 ATPase provide the most visually striking image of how kinetic asymmetry allows coupling between two processes, in this case rotation and catalysis. Using the known crystal structures of the enzyme Mukherjee and Warshel calculated[103,104] the energy of the molecule at many different constrained rotational and chemical states, and interpolated between these to generate a two-dimensional energy landscape (Fig. 4a) in which a low energy valley, shown in blue, runs from the upper left to lower right corner, labelled $\mathcal{F}_R$ and $\mathcal{F}$, respectively. The selection to favor the cycles $\mathcal{F}_R$ and $\mathcal{F}$ over the cycles $\mathcal{B}_R$ and $\mathcal{B}$ is due to the structure of the enzyme – the energy surface can be described as a sculpted landscape on which the system undergoes diffusion. The vast majority of the diffusive traffic will occur back and forth in the zig-zag blue energy valley, where chemical and mechanical transitions are interleaved[85] with one another, but at equilibrium there will be no preference for a trajectory from upper left to lower right vs. from lower right to upper left hand corner. Directionality arises if the chemical potential $\Delta\mu_{SP} = \mu_{ATP} - (\mu_{ADP} + \mu_{Pi}) \neq 0$ to favor trajectories from top to bottom relative to trajectories from bottom to top. The directionality can be given in terms of the ratchetting equation Eq. (12), with the relations $\frac{\pi_{\mathcal{F}}}{\pi_{\mathcal{F}_R}} = \frac{\pi_{\mathcal{B}}}{\pi_{\mathcal{B}_R}} = e^{\Delta\mu_{SP}/RT}$, $\frac{\pi_{\mathcal{F}}}{\pi_{\mathcal{B}}} = e^{\ln q}$, and $\frac{\pi_{u_1}}{\pi_{u_{1,R}}} = \frac{\pi_{u_2}}{\pi_{u_{2,R}}} = 1$. The mechanism involves interleaving the mechanical rotations with the chemical transitions of hydrolysis, binding ATP, and departure of ADP. The striking picture of the zigzag energy well constraining diffusion to give rise to coupling between ATP hydrolysis and positive rotation of the FoF1 ATP synthase inspired effort to design a synthetic electric rotor based on a catenane[20].

The basic idea was to construct a catenane rotor similar to that shown in Fig. 3b), but with a directional[105] redox driven pumping cassette (Fig. 4b top) developed in the context of a synthetic rotaxane pump[28]. Initial effort with a [2] catenane did not yield sufficient pumping to result in detectable directionality. Incorporating a second mobile ring on the large circular track resulted[20] in a [3]-catenane where the interactions between the two small rings play the role of gearing in macroscopic machines. Kinetic gating due to the isopropyl phenyl speed bump (green) and the pyridinium electrostatic barrier (blue) flanking a triazole moiety (magenta) allowed a kinetic mechanism in which the rings rotate passing over the substituents in the order → green → magenta → blue →green → where every two cycles of oxidation and reduction produces on average 0.8 complete cycles of each ring. Quantum mechanical calculations confirmed the heuristic understanding devoloped for explaining the directionality of the rotor. The arrows on the 2-D plot of the position of the two rings are the optimal trajectories based on the energetic calculations, and the purple and blue dots represent the most stable states in the reduced and oxidized forms, respectively. The kinetic asymmetry arises because of the different relative barrier heights due to the IPP and Py+ groups in the oxidized state, where the barrier of IPP is smaller than that over Py+, vs. the reduced state where the barrier over the Py+ is smaller than that over the IPP.
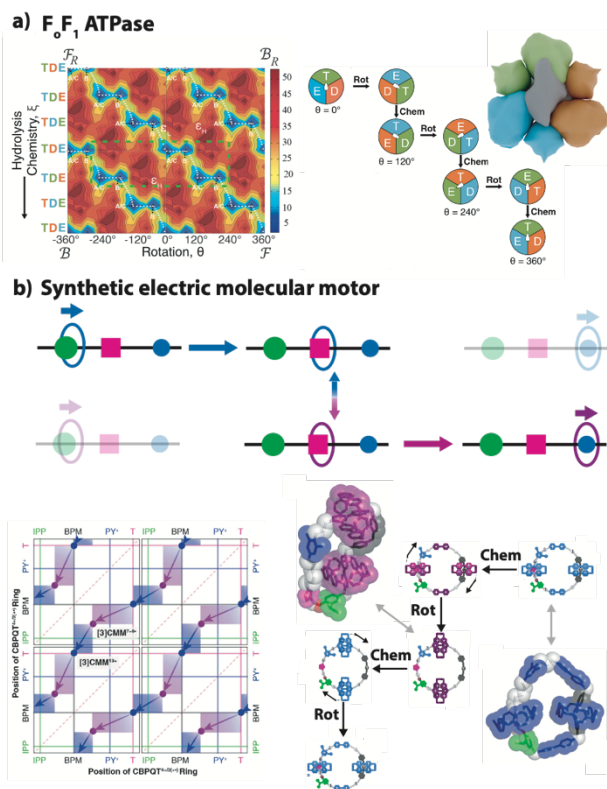
a) F_oF_1 ATPase

b) Synthetic electric molecular motor

Figure 4 Coupling in molecular machines can be visuazed in terms of 2-D energy landscapes. A) Energy landscape for the F1 ATPase. The zigzag valley running from upper left to lower right defines the coupling. A downward tendency due to an excess of ATP couples to rotational diffusion to the right (positive rotation). The mechanism by which hydrolysis of ATP promotes rotation is best described as mass action. The molecule is in mechanical equilibrium at every instant. The ATP driven rotational catalysis by the F1 ATPase works by diffusion on a sculpted energy landscape biased by virtue of the fact that binding ATP is faster than binding ADP when the binding site is unoccupied – mass action. b) a synthetic molecular rotor, the design of which was inspired by the FoF1-ATPase. The basic idea was to use the directionality imbued by the pumping cassette shown at the top of Fig. 4 b). It turned out that the rotation was not significant with a [2]-catenane, leading to use of a [3]-catenane, where the interactions between the two mobile rings give rise to effective "gearing" and nearly perfect precision, with .8 cycles of each ring per cycle of reduction -> oxidation -> reduction.

4. Conclusion

Consider a reaction in chemical equilibrium where the concentrations of the species in the reaction $A \underset{k_{AA'}}{\overset{k_{AA'}}{\rightleftharpoons}} A'$ obey the equilibrium relation $\frac{[A']_{eq}}{[A]_{eq}} = \frac{k_{AA'}}{k_{A'A}} = K_{AA'}$. If external energy is added to the system the reaction can be shifted to a different ratio between the concentrations. Heating the system always results in a ratio that is closer to unity, i.e., heating favors the higher free-energy state. Similarly, if the reacting system can absorb light, illumination favors the high free-energy state and brings the ratio closer to unity. By an intelligent arrangement of thermally allowed and photochemically allowed reaction steps, light driven molecular motors and rotors can and have been designed. Both light and heat can result in a situation where the high energy form has nearly an equal concentration as the low energy form, and when incorporated into a cyclic chemical network, the energy absorbed can be used to drive directional motion. The directionality is determined by the direction in which the ground state processes are exergonic – i.e., by a power-stroke.

Most biomolecular motors are driven, however, not by heat or light but by coupling to some chemical reaction, e.g., ATP hydrolysis that provides a certain amount of energy, $\delta\varepsilon \ (= \Delta\mu_{ATP})$ for each turnover. The natural question is „in which direction is the reaction $A \rightleftharpoons A'$ driven"? Does ATP hydrolysis always favor the higher free-energy form, or does it depend on the mechanism, and if the latter, on what properties of the reaction between A and A' does it depend? The answer to this question can be undersood by taking a linear combination of the two pair of rate constants thermodynamically consistent with the effect of the input energy $\delta\varepsilon$ assuming it to act stoichiometrically, Eqs. 2a) and 2b), as shown in Eq. (3). If the ratio

$\frac{k_{A'\to A}^{(1)}}{k_{A'\to A}^{(2)}} = e^{-\delta\varepsilon/RT}$ of net rate constants is equal the equilibrium constant, $\frac{k(A\to A')}{k(A'\to A)} = K_{AA'}$ despite the presence of continual dissipation when $\delta\varepsilon \neq 0$. This condition is known as kinetic symmetry, the breaking of which to achieve kinetic asymmetry[48] was discussed in 1989 as the key determinant of whether an enzyme could use energy driven fluctuations to perform chemical, osmotic, or mechanical work on the environment. Unlike the ratio of rate constants for a light driven process, which lies between $K_{AA'}$ and 1, the ratio of net rate constants for pumping by external modulation or chemical catalysis lies between $K_{AA'}e^{-\delta\varepsilon/RT}$ and $K_{AA'}e^{+\delta\varepsilon/RT}$. As with light/heat driven systems, incorporation of A $\rightleftarrows$ A' into a cyclic chemical reaction network can lead to directed motion.

When transitions that can be pumped are arranged to give a kinetic cycle[93], e.g., ~A$\rightleftarrows$B$\rightleftarrows$C$\rightleftarrows$A~ a steady-state can be achieved where there is a continual cyclic flux in one direction or the other – a directionality. Onsager pointed out that at equilibrium an additional constraint known as detailed balance holds. According to the principle of detailed balance each transition is pairwise balanced at equilibrium, e.g. $k(A\to B)[A]_{eq} = k(B\to A)[B]_{eq}$, etc. and so there is no directional cycling. Away from equilibrium detailed balance can be broken, a fact that is important not only for understanding biomolecular functions such as motility, adaptation, assembly, kinetic proofreading, etc. but possibly also for higher level function such as cognition[106].

Note that while being away from equillibrium is necessary to break detailed balance it is not sufficient. As pointed out by Astumian et al.[48], detailed balance holds even away from equilibrium if the system is kinetically symmetric. The cycle probability $\frac{\pi(A\to B\to C\to A)}{\pi(A\to C\to B\to A)} = \langle e^{\mathcal{W}_{AB}/RT}\rangle\langle e^{\mathcal{W}_{BC}/RT}\rangle\langle e^{\mathcal{W}_{CA}/RT}\rangle$ is unity if the product of the exponential averages of the energy exchanged with the environment is one (e.g., $\langle e^{\mathcal{W}_{AB}/RT}\rangle = 1$ and $\langle e^{\mathcal{W}_{BC}/RT}\rangle = \langle e^{\mathcal{W}_{CA}/RT}\rangle^{-1}$), even if there is continual dissipation. Not only is there no net flux, even the fluctuations of the states shows no signature of the dissipation if the system is kinetically symmetric The performance of many of lifes functions requires the breaking of detailed balance, which in turn requires both dissipation (production of entropy) and kinetic asymmetry.

The ubiquitous claim that directionality of a mechanism specified in terms of a sequence of states of a molecular machine is given by a „thermodynamic driving force" or „cycle affinity" is simply wrong. A thermodynamic driving force can be defined only for a sequence of states in which the reaction channel[70] for each transition is also specified. To calculate the overall directionality it is necessary to obtain the kinetically weighted average over all possible combinations of reaction channels in the sequence, where every pumped process has at least two possible reaction channels. For the cycles in Fig. 1 where we take the minimum of two reaction channels for each pumped process the directionality is given in Eq. (4), or equivalently Eq. (7).

The fundamental theory of trajectory thermodynamics for molecular motors and pumps is based on kinetic asymmetry and microscopic reversibility. This theory is not difficult but applying the insights gained from the theory requires that many long-held ideas about the function of molecular machines must be abandoned. For example, it has become almost obligatory to begin and end discussions of molecular machines with the reiteration that they "operate far from equilibrium" but what does this mean? A molecular machine in solution is, in the only sense meaningful for an individual object, in mechanical equilibrium at every instant[58-60]. Violent kicks[52], judo throws[107], and the like are not sensible descriptions of molecular motions in solution. Instead, the motion is best described as diffusion on a sculpted energy landscape, where evolution in the case of biomolecular machines, or the chemist in the case of synthetic molecular machines, is the sculptor.

The distance from chemical equilibrium amongst the states for a specific reaction is parametrized by the non-equilibrium pump equality Eq. (11). The pumping equality[49] hinges on an understanding of non-equilibrium processes in which thermal noise is the proximal cause of the process. Switching of an energy surface, either by external changes (e.g., adding reductant or oxidant) or by catalysis of a chemical reaction, provides the ultimate source of energy necessary for achieving directed motion but thermal noise is an essential ingredient to the directed motion, rather than a hindering nuisance as it is in attempts to actuate miniaturized versions of macroscopic machines. These Brownian motor mechanisms[87,108-111] exploit rather than fight against thermal noise. They rely on kinetic asymmetry to provide directionality to the overall process. The key perspective associated with the Brownian information and energy ratchet models[56] is that the directionality is governed by the kinetic symmetry of the transition states for binding/release of substrate and product and is independent of the free energies of the states of the motor. Note that it is not the case that thermal fluctuations are converted to work as sometimes claimed. Instead, energy ratchets combine the incessant thermal noise with input energy in the form of random fluctuations

that destroy correlations between the state of the molecule and a transition probability that would be present at equilibrium resulting in directionality and the performance of work. Information ratchets use thermal noise combined with energy released by catalysis of an exergonic reaction to increase the correlations between the state of the system and the likelihood of a transition to achieve directionality and the performance of work. Energy and information ratchets have been described in terms of Smoluchowski and Maxwell demons[112,113], respectively (see TOC graphic). A Smoluchowski demon is blindfolded and perturbs the system randomly, where both the energy of an intermediate and the relative barrier heights must fluctuate. A Maxwell demon, on the other hand gathers information about the state of the system and uses this information to decide when to open and close gates (perturb the relative barrier heights). Both light driven[114] and chemically driven[115] information ratchets have been synthesized and characterized. Application of information thermodynamics is a promising avenue for theoretical exploration of non-equilibrium chemistry[116], and combination with trajectory thermodynamics is likely to yield important new insights.[117,118]

The idea of a configurational or conformational transition known as the power stroke[54,55] that involves a large free-energy change and significant displacement has a long history in the development of understanding biomolecular motors. The hypothesis associated with this model[52] is that the preferred direction of motion is, of necessity, that in which the power-stroke is exergonic. Application of this idea has proven extraordinarily fruitful in the design[4,9] of synthetic light driven rotors. The first synthetic catalysis driven rotor[21], however, provided a shock for the field – the direction of rotation was opposite to that predicted by the power stroke model, but was correctly interpreted by the Brownian information ratchet[45,41] model where the direction of motion is governed by the free-energies of the transition-states - i.e., by kinetic gating. The necessary ingredient was kinetic asymmetry. Similarly, while studies that focus on individual transitions may reveal a "structural origin" for the power-stroke[55], when viewed holistically in terms of the complete mechanochemical cycle[16,95] the free-energy difference between the pre- and post-power-stroke states is seen to play no role whatsoever in determining the directionality of the motor. The sole determinate of the directionality is the kinetic asymmetry parametrized by $e^{\ln q}$. For the rotor[21] of Wilson et al. the kinetic asymmetry is $e^{\ln q} \approx 0.25$, consistent with the motor moving in the direction in which the „power-stroke" is endergonic. The detailed understanding of the different design principles for light-driven rotors, for which the directionality is governed by a power stroke, and for catalysis-driven rotors that operate as information ratchets and for which the directionality is governed by kinetic asymmetry $e^{\ln q}$ has significantly stimulated development of new molecular machines.

A key consideration for developing molecular machines of the future will be design of mechanisms for allosteric interactions[119,120] where the mechanical state of the motor controls the chemical reactivity to enforce an ordered cycling through mechanical states when the chemical reaction is maintained away from equilibrium. These and other ideas[121] stemming from an understanding of the non-equilibrium pumping equality[49] and and the key idea that away from equilibrium transitions between any two states can occur by at least two channels. Development of mechanisms to control the kinetic asymmetry between the several reaction channels in non-equilibrium systems will certainly play an important role in the design of molecular machines and their incorporation into larger structures[36,37,38,39] in the future.

Acknowledgements

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