

Phosphorylation Energy Hypothesis: Open Chemical Systems and Their Biological Functions

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Abstract

Biochemical systems and processes in living cells generally operate far from equilibrium. This review presents an overview of a statistical thermodynamic treatment for such systems, with examples from several key components in cellular signal transduction. Open-system nonequilibrium steady-state (NESS) models are introduced. The models account quantitatively for the energetics and thermodynamics in phosphorylation-dephosphorylation switches, GTPase timers, and specificity amplification through kinetic proofreading. The chemical energy derived from ATP and GTP hydrolysis establishes the NESS of a cell and makes the cell—a mesoscopic–biochemical reaction system that consists of a collection of thermally driven fluctuating macromolecules—a genetically programmed chemical machine.

ATP: adenosine triphosphate

Motor protein: a single protein, by hydrolyse ATP, is able to move against force and converts chemical energy to mechanical force

Signal transduction: cells generate, process, and deliver information via the biological activities of proteins, in terms of chemical reactions

Nonequilibrium steady state (NESS): a chemical system with all the concentrations and fluctuations being stationary; the system has fluxes and dissipates heat

Thermodynamics: the theoretical basis of any machine, the dynamics in terms of transformations of various form of energies, including entropy

ADP: adenosine diphosphate

P_i: orthophosphate

1. INTRODUCTION

Biochemical reaction systems in a living cell are open systems. They exchange materials with their environment, and they consume chemical energy, usually in the form of adenosine triphosphate (ATP) hydrolysis, accompanied with dissipating heat. In current biochemical research, investigators are directing great effort toward elucidating biochemical complexity. But complexity is only one aspect of living systems: No matter how complex a biochemical reaction system is, if it is left alone in a closed test tube, it will gradually decay to become a pile of dirt. In terms of physical chemistry, a closed system has no life (1). Current cellular signaling research could benefit from a rigorous physiochemical approach to the functional biochemical systems, with more attention paid to the continuous supply of energy and the removal of waste. This is true for all living systems, even those as simple as a single motor protein (2, 3).

It is intuitively obvious that living biochemical systems need free energy. Schrödinger (4) made it clear that the organization of living matter requires negative entropy. But how is energy actually utilized and what are its specific biological functions? In textbooks, the functions of ATP hydrolysis inside a cell are said to be (*a*) biosynthesis, (*b*) ionic and neutral molecular pumping, and (*c*) mechanical movement. They are known collectively as the three major energy sinks at the cellular level (5). This view, however, is too limited and too mechanical. In fact, researchers have suggested that free energy liberated from the phosphorylation and dephosphorylation cycles of proteins might be used to correct errors in biomolecular recognition (6) and to improve robustness in cell development (7). In other words, energy may play an important role in biological information processing and biochemical signal transduction. In biochemical reactions involved in signaling, high-grade chemical energy is reduced to low-grade heat. The energy involved in processing information must be explained in terms of entropy production—the central concept in nonequilibrium steady-state (NESS) thermodynamics (8, 9).

How much free energy is dissipated in normal cellular-information processing? This is an important question to ask, but surprisingly, I have not been able to find much data on this. Classic thermodynamics has taught us how to estimate the amount of chemical energy utilized in doing work, which has to do with the concept of efficiency. In the age of information, we recognize that free energy is needed not just to do work, but also for error correction and signal processing. This is an aspect that has not been widely appreciated but should be addressed in both cellular biology and physical chemistry.

The available energy relevant to a normal living cell is from the sustained high concentration of ATP (~1 mM) and low concentrations of adenosine diphosphate (ADP) (~10 μM). With an equilibrium constant of 4.9×10^5 M for ATP hydrolysis and a P_i (orthophosphate) concentration of ~1 mM, the phosphorylation potential in a normal cell is approximately 12 kcal mol⁻¹ (10). The phosphate bond of the ATP molecule per se does not provide the energy; as Nicholls & Ferguson (11) said, “[t]he Pacific Ocean could be filled with an equilibrium mixture of ATP, ADP and P_i, but the ATP would have no capacity to do work.” For example, when a cardiac myocyte experiences ischemia, its ATP concentration goes down while its ADP

concentration goes up, and the cellular energy is decreased (12). It is not unreasonable to suggest that cellular processes such as cell cycle, differentiation, apoptosis, and cancer development are regulated by cellular energy.

Currently, there is great enthusiasm for quantitative approaches and mathematical modeling in biology. But if we peek beneath the surface of this passion, we are struck with a compelling question: How is it possible to develop mathematical models of cellular processes such as gene regulation and signal transduction if even the underlying basic physical chemistry is still not in hand?

This review focuses on presenting a theory of NESS with fluctuations and demonstrating how such a mesoscopic theory can be useful in understanding living systems from a physical chemistry standpoint. An introductory account of the theory of NESS has already appeared in Reference 13, and Reference 3 summarizes the application of this approach to motor proteins, which can be thought of as an archetype.

This review is organized as follows: Section 2 illustrates the basic idea of NESS and its relevance to cellular biochemistry. Applying the ideas and modeling tools from Section 2, Sections 3–5 focus on three key biochemical systems and subject them to nonequilibrium thermodynamic analyses. These analyses together show the possible new biological insights one obtains from physiochemical studies of open biochemical systems far from equilibrium and establish the importance of energy supply in biochemical signal transduction inside living cells. Finally, Section 6 provides some summary remarks.

2. EQUILIBRIUM AND NONEQUILIBRIUM STEADY STATES

Chemical equilibrium is a steady state, by which I mean the concentrations of all the chemical species are constant macroscopically but with stationary fluctuations in a statistical sense. Here I emphasize the statistical nature of a chemical equilibrium: For an equilibrium system with only a few copies of some molecules, thermal fluctuations are significant. This is best understood in a single-molecule experiment in which the probability for each state of an ever-fluctuating molecule is independent of time (14).

However, not every chemical steady state is an equilibrium. The fundamental difference between an equilibrium steady state and an NESS is that the concentrations (or probabilities) are maintained constant by detailed balance in the former but by circular balance in the latter (**Figure 1**). There is no net flux in any reaction of a chemical equilibrium, whereas there are necessarily sources and sinks for a chemical NESS. The consequences of this distinction are severalfold. First, a chemical NESS can exist only in an open system in which reactions are driven from sources to sinks between which there must be a chemical-potential difference. If there is no chemical-potential difference between two species, then there is no distinction between them as a source and a sink. In this case, the chemical system is in an equilibrium following the grand canonical ensemble of Gibbs (15). Second, a driven chemical reaction with flux generates heat (16). Third, the temporal fluctuations of the chemical species in a cyclic reaction are time irreversible (17). The breakdown of the temporal symmetry leads to the possibility of chemical oscillations (18) and other more complex behavior in space and time (19) in biochemical systems.

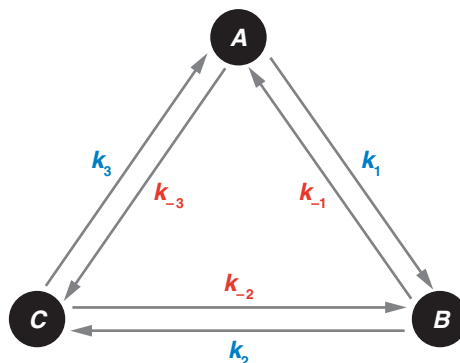


Figure 1

Simple, unimolecular chemical reaction cycle. In a closed system, the principle of detailed balance dictates that $k_1 k_2 k_3 / (k_{-1} k_{-2} k_{-3}) = 1$. Noting that (Equation 2), $\Delta\mu_{AB} = k_B T \ln(k_{-1}[B]/k_1[A])$, $\Delta\mu_{BC} = k_B T \ln(k_{-2}[C]/k_2[B])$, and $\Delta\mu_{CA} = k_B T \ln(k_{-3}[A]/k_3[C])$. Thus, we have $\Delta\mu_{AB} + \Delta\mu_{BC} + \Delta\mu_{CA} = 0$ for all time in a closed system.

2.1. Detailed Balance in Closed Systems

The idea of detailed balance, which distinguishes a chemical equilibrium from an NESS, was already present, although implicit, in the original work of Boltzmann's kinetic theory (see Reference 20, section 8.2; Boltzmann had originally made a mistake, which was pointed out by H.A. Lorentz). Lewis (21) formally proposed the concept of detailed balance in 1925. In introductory chemistry, professors teach detailed balance as the so-called thermodynamic box that has to be obeyed because of the very existence of a unique equilibrium constant for each and every chemical reaction.¹ More importantly, the equilibrium constant K_{eq} for a reaction, for example,



is directly related to the standard-state chemical-potential difference of the chemical reaction: $\Delta\mu^\circ = -k_B T \ln K_{eq}$. Based on the law of mass action, we can write the forward- and backward-reaction fluxes as $\mathcal{J}_+ = k_+[A][D]$ and $\mathcal{J}_- = k_-[B][E]$, where $k_+/k_- = K_{eq}$. Less widely shown, however, is the chemical-potential difference, $\Delta\mu$, between the reactants and the products, as a function of the fluxes:

$$\Delta\mu = \Delta\mu^\circ + k_B T \ln \frac{[B][E]}{[A][D]} = k_B T \ln \frac{\mathcal{J}_-}{\mathcal{J}_+}. \quad (2)$$

Equation 2 shows that the chemical equilibrium (i.e., $\Delta\mu = 0$) dictates that $\mathcal{J}_+ = \mathcal{J}_-$ (i.e., detailed balance). The net-reaction flux $\mathcal{J} = \mathcal{J}_+ - \mathcal{J}_- = 0$ is sufficient and necessary for a chemical equilibrium.

¹The assumption here is that the chemical reaction systems in aqueous solution are overdamped, and hence all the dynamic variables are even according to Marchlup & Onsager (22, 23). For underdamped systems with both even and odd variables, motion such as superconducting in a magnetic field could break the detailed balance without energy dissipation (24).

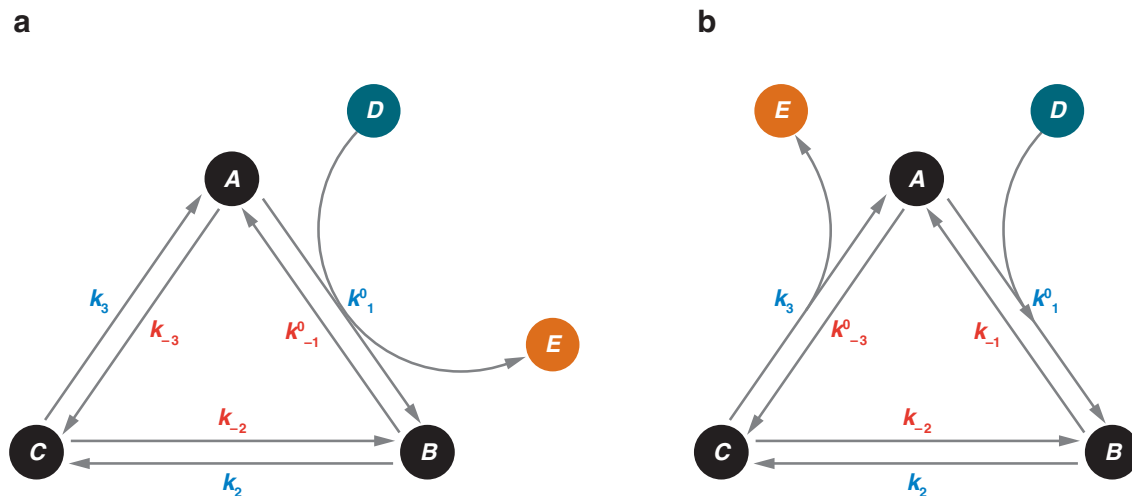


Figure 2

Cyclic enzyme reactions with substrates D and E can be mapped into the unimolecular cycle in **Figure 1** in terms of pseudo-first-order rate constants: $k_1 = k_1^0[D]$ and $k_{-1} = k_{-1}^0[E]$ in *a*, and $k_1 = k_1^0[D]$ and $k_{-3} = k_{-3}^0[E]$ in *b*. If the species D and E are in equilibrium, then we have $\gamma = k_1 k_2 k_3 / (k_{-1} k_{-2} k_{-3}) = 1$. However, if $[D]$ and $[E]$ are sustained under nonequilibrium conditions in an open system, then the chemical-potential difference between D and E , $\Delta\mu_{ED} = k_B T \ln \gamma$, is not equal to zero. The difference $\Delta\mu_{ED} > 0$ drives a cyclic flux in the reactions $A \rightarrow B \rightarrow C \rightarrow A$.

For a set of reactions in a closed system with detailed balance, both flux \mathcal{J} and chemical-potential difference $\Delta\mu$ are zero for each and every reaction in the steady state. We can in fact mathematically prove that the equilibrium steady state is unique and globally attractive (25). Then, if there is a reaction cycle as shown in **Figure 1**,

$$\frac{[B]^{eq} [C]^{eq} [A]^{eq}}{[A]^{eq} [B]^{eq} [C]^{eq}} = \frac{k_1 k_2 k_3}{k_{-1} k_{-2} k_{-3}} = 1. \quad (3)$$

This is what is known as the thermodynamic box.

Now let us consider the kinetic schemes shown in **Figure 2**. We can let A , B , and C be three conformations of a single enzyme, and D and E be substrates. If the concentrations of the substrates are significantly greater than the enzyme, then we can effectively introduce pseudo-first-order rate constants. It is easy then to show that chemical equilibrium between species D and E is equivalent to Equation 3. We can take **Figure 2a**, for example:

$$\frac{k_1^0 k_2 k_3 [D]^{eq}}{k_{-1}^0 k_{-2} k_{-3} [E]^{eq}} = \frac{k_1^{eq} k_2 k_3}{k_{-1}^{eq} k_{-2} k_{-3}} = 1. \quad (4)$$

The same result holds for **Figure 2b**. Hence, from the standpoint of the cyclic enzyme reaction, we have to consider only the unimolecular reaction cycle in **Figure 1**. Reaction cycles are fundamental to biochemical network kinetics (16, 26).

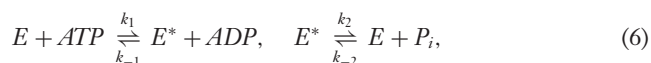
2.2. Nonequilibrium Steady States in an Open System

The situation is quite different in an open system. Let us take the kinetics in **Figure 2** as an example. If the enzyme reactions are in open systems with concentrations of D and E sustained by an external agent, then

$$\Delta\mu_{ED} = k_B T \ln \gamma = k_B T \ln \frac{k_1 k_2 k_3}{k_{-1} k_{-2} k_{-3}} \quad (5)$$

is the chemical driving force for cyclic reactions $A \rightarrow B \rightarrow C \rightarrow A$. Even though the detailed kinetics are different in **Figures 2a,b**, from an enzyme kinetic standpoint, the fundamental difference is whether $\gamma = 1$, that is, whether the reaction loop in **Figure 1** satisfies detailed balance or whether the system is closed or open. A closed system tends to be an equilibrium, whereas an open system tends to be an NESS.

Let us now consider a more realistic pair of biochemical reactions in which an enzyme, E , is phosphorylated and then dephosphorylated:



in which E^* is the phosphorylated form of E . We assume the concentrations of ATP , ADP , and P_i are held at constant c_T , c_D , and c_P , a reasonable assumption for a normal physiological intracellular environment and an idealization for open chemical systems. Then the kinetic equations based on the law of mass action are

$$\frac{d[E]}{dt} = -\frac{d[E^*]}{dt} = -k_1[E]c_T + k_{-1}[E^*]c_D + k_2[E^*] - k_{-2}c_P[E]. \quad (7)$$

The steady-state concentrations for E and E^* are easily obtained:

$$[E]^{ss} = \frac{k_{-1}c_D + k_2}{k_1c_T + k_{-2}c_P + k_{-1}c_D + k_2}, \quad [E^*]^{ss} = \frac{k_1c_T + k_{-2}c_P}{k_1c_T + k_{-2}c_P + k_{-1}c_D + k_2}.$$

There is also a steady-state cycle flux,

$$\mathcal{J}^{ss} = \frac{k_1 k_2 c_T - k_{-1} k_{-2} c_D c_P}{k_1 c_T + k_{-2} c_P + k_{-1} c_D + k_2}. \quad (8)$$

We should note that the free energy of one ATP hydrolysis is

$$\Delta\mu_{DT} = k_B T \ln \frac{k_{-1} k_{-2} c_D c_P}{k_1 k_2 c_T}, \quad (9)$$

which is usually sustained by the constant levels of c_T , c_D , and c_P in a living cell. More interestingly, we have

$$-\mathcal{J}^{ss} \times \Delta\mu_{DT} \geq 0, \quad (10)$$

where the equal sign holds true if and only if $\mathcal{J}^{ss} = 0$ and $\Delta\mu_{DT} = 0$. The product of \mathcal{J}^{ss} and $\Delta\mu_{DT}$ in Equation 10 is the ATP hydrolysis energy per unit time, which is also the rate of heat dissipation of the chemical reaction system in its NESS. There is a close analog between this equation and the fact that current multiplied by voltage equals power in an electrical circuit. The inequality is in fact a statement of the second law of thermodynamics: With only a single-temperature bath T , we can only continuously convert chemical work to heat, but not the reverse. If the reverse were possible, then we would have a chemical perpetual-motion machine of the second kind (27).

2.3. Fluctuating Nonequilibrium Steady States of an Open System and Its Uniqueness

With the understanding that an NESS is a state of an open system with fluctuations, we can now discuss NESSs of open systems more generally. Here I focus exclusively on chemical reaction systems.² There are three widely used classes of stochastic kinetic equations for characterizing fluctuations. The first class, Hill's (16) approach, uses linear rate equations for a unimolecular reaction network. The second class, which has its origin in the Kramers' theory and polymer theory (28, 29), uses the general Brownian dynamic equation for single macromolecules in a phase space with an internal energy landscape. We have recently developed this theory further for open single macromolecules in NESSs (30, 31). The third class, widely known as the Gillespie algorithm (32), uses the chemical master equation (CME) for a nonlinear-chemical reaction network. All three classes of models are well studied in the mathematical theory of Markov processes (23, 33). They are known as continuous-time Markov chains, diffusion processes, and birth-death processes, respectively. One of the most important results from the mathematical study is that with quite general conditions, all these Markov processes have a unique stationary long time limit (i.e., a steady state). In chemical terms, this means most of these reaction systems are self-organizing: No matter what the initial state of a system is, after a long time it approaches a unique steady state with stationary fluctuations. Under this definition, a system with bistability simply has a bimodal steady-state probability distribution, and a system with sustained chemical oscillation has a steady-state distribution concentrated along a closed loop in concentration space (34).

As with the equilibrium steady state of a closed system, the unique NESS of an open system is a function of the conditions in which the system is situated. In addition to the usual temperature, pressure, and solvent conditions, an NESS depends in particular on the nature of the sources and sinks. For example, a specific chemical species as a source can be at a fixed concentration or have a constant rate of production (flux). This has a close analogy to the two types of ideal batteries in electrical circuit theory: the constant voltage and the constant current power supplies. A real battery, of course, is in between these two limits, as is a real biochemical source, or sink, in a living cell. Nevertheless, idealization is essential in theoretical studies.

For every open biochemical system, a corresponding closed system can be obtained by shutting off the sources and the sinks. This corresponding system is an important reference for the open system and its thermodynamics.

2.4. Energy Exchange with Molecular Number Conservation

One of the simplest open biochemical reactions is an enzyme-catalyzed reaction with substrate and product concentrations held constant (**Figure 2**). The reaction is open because the species *D* and *E* are held constant as a source and a sink of the system. There are material exchanges. However, from an enzyme-kinetic standpoint, it is

CME: chemical master equation

²Other, nonchemical examples of NESS are forced-dissipative fluids and electrical circuits with batteries.

Kinetic proofreading:

biochemical specificity can be modulated by purely kinetic means without changing molecular structures

Stochastic processes:

dynamic processes for which one cannot say when and where, but when and the probabilities of being where

even simpler to consider the enzyme cycle $A \rightleftharpoons B \rightleftharpoons C \rightleftharpoons A$ with unimolecular rate constants. In such a representation, material exchange becomes implicit, and the consequence of the open system is the chemical energy input $\gamma \neq 1$.

From the enzyme-kinetic perspective, the system now has a constant number of molecules. Assuming there are N enzymes, then the total number of molecules in states A , B , and C ($n_A + n_B + n_C = N$) is constant. The fluctuations in the molecular numbers (n_A , n_B , and n_C) are not independent. In fact, the joint probability follows a multinomial distribution

$$P(n_A, n_B, n_C) = \frac{N!}{n_A! n_B! n_C!} p_A^{n_A} p_B^{n_B} p_C^{n_C}, \quad (11)$$

where p_X ($X = A, B, C$) satisfy the stochastic model for a single enzyme in terms of a master equation.

Hill (16, 35) has used extensively this approach to open systems with single enzyme molecules. It is also the mathematical basis of Hopfield's (6) theory of kinetic proofreading, and recent work on chemical models of motor proteins (3, 36, 37) and stochastic Michaelis-Menten kinetics (38, 39).

2.5. Grand Canonical Systems with Material Exchange

Not all open biochemical reactions can be made implicit by the conservation of the number of molecules. Let us consider the simple example



If an NESS is sustained by constant concentrations of A and B , then the number of X molecules fluctuates. The probability of having n X molecules, $p_n(t)$, satisfies

$$\frac{dp_n(t)}{dt} = -(k_{-1} + k_2)np_n + (k_1n_A + k_{-2}n_B). \quad (13)$$

The steady-state p_n from Equation 13 is a Poisson distribution,

$$p_n = \frac{\lambda^n}{n!} e^{-\lambda}, \quad (14)$$

where $\lambda = \frac{k_1n_A + k_{-2}n_B}{k_{-1} + k_2}$ is the mean number of X . If N in Equation 11, the total number of molecules in the system, is not a constant but instead fluctuates following a Poisson distribution, then

$$P(n_A, n_B, n_C) = \sum_{N=0}^{\infty} P(n_A, n_B, n_C | N) P_N = \prod_{X=A,B,C} \left(\frac{(\lambda p_X)^{n_X}}{n_X!} e^{-\lambda p_X} \right). \quad (15)$$

That is, in an open system with a fluctuating total number of molecules, the number of molecules in each state is Poisson distributed. The situation is completely analogous to the grand canonical ensemble in equilibrium statistical mechanics. (See Reference 40 for more discussion.)

2.6. Stochastic Systems with Nonequilibrium Thermodynamics

To study the thermodynamics of a mesoscopic NESS, one needs to have a general theory of nonequilibrium thermodynamics with fluctuations. In recent years, there have been two approaches for establishing the basic, stochastic formalism for such a theory. Rubí and coworkers (41) started their approach with basic thermodynamic considerations in probabilistic terms. Our own work (3, 30, 31), which was motivated by the theory of molecular motors, is based on chemical kinetic equations, most notably the Markov models for single molecules (2, 36, 38, 42, 43) and the CMEs for nonlinear chemical reactions (25, 34). As mesoscopic theories, both approaches focus on the probability density function, $P(x, t)$, in a phase space and introduce entropy, and free energy, associated with the distribution

$$S[P(x, t)] = -k_B \int P(x, t) \ln P(x, t), \quad F[P(x, t)] = \int P(x, t) \mu(x, t) dx, \quad (16)$$

where $\mu(x, t) = U(x) + k_B T \ln P(x, t)$ is the local chemical-potential function and $U(x)$ is the internal energy function. $S[P(x, t)]$ and $F[P(x, t)]$ indicate that the entropy and free energy are functions of the probability distribution $P(x, t)$. Both approaches have led to essentially similar thermodynamic formalisms in the form of a Fokker-Planck equation, in which there is a linear relationship between the thermodynamic flux and force. In fact, both approaches yield

$$T \frac{dS}{dt} = -bdr + epr, \quad (17)$$

where bdr is the entropy flux (i.e., isothermal heat dissipation rate), epr is the entropy production rate, and $\mathcal{J}(x, t)$ below is the nonequilibrium flux in the phase space:

$$bdr = \int \nabla \cdot (\mathcal{J}(x, t) \mu(x, t)) dx, \quad epr = - \int \mathcal{J}(x, t) \cdot \nabla \mu(x, t) dx, \quad (18)$$

$$\frac{\partial P(x, t)}{\partial t} = -\nabla \cdot \mathcal{J}(x, t). \quad (19)$$

If we assume that $\mathcal{J} \propto -\nabla \mu$, then we arrive at a Fokker-Planck equation (41), shown in Equation 20 below. For mesoscopic systems with discrete states, however, the Markov flux between states A and B , $\mathcal{J}_{AB} = k_{AB} P_A - k_{BA} P_B$, is not linearly proportional to $\Delta \mu_{AB} = k_B T \ln(k_{BA} P_B / (k_{AB} P_A))$. Still, when integrations are replaced by summations, Equations 16–18 apply to discrete master-equation systems (16, 44), which means the thermodynamic formalism is also applicable to nonlinear, far-from-equilibrium chemical reaction systems.

2.7. Single Macromolecules as Open Systems

There are two stochastic modeling approaches to single macromolecules. One is based on phenomenological rate equations for discrete conformational states of a molecule, and the other is based on a continuous energy-landscape description of a macromolecule as a polymer (45). Both approaches have been widely used in physical chemistry and biophysics.

By applying the continuous energy–landscape approach (i.e., Kramers’ theory) to a single macromolecule in NESSs, we developed a theory of stochastic macromolecular mechanics (SM₃) (30, 31, 46). To model an open system, we introduced a driving force in addition to the internal conformational energy $U(x)$. Hence, the total force in the Fokker–Planck equation is $F(x) = -\nabla U(x) + G(x)$, where $G(x)$ usually does not have a potential ($\nabla \times G(x) \neq 0$). The nonpotential force represents the breakdown of detailed balance (47) owing to implicit sources and sinks. When they are shut off, $G(x) = 0$, and $U(x)$ represents the energy landscape of the corresponding closed system.

The Fokker–Planck equation for an open system has the standard form

$$\frac{\partial P(x, t)}{\partial t} = \nabla \cdot \left\{ \frac{P(x, t)}{\eta(x)} [k_B T \nabla \ln P(x, t) - F(x)] \right\}. \quad (20)$$

What is the relation between this dynamics equation and the laws of thermodynamics? Can thermodynamics be rigorously established from it? It turns out, if we introduce the correct definitions for various thermodynamic quantities, then Equation 20 in fact contains Equation 17, and more.

We can summarize the thermodynamics in the following equations. Let us recall that the system is undergoing stochastic dynamics X_t in phase space with probability distribution $P(x, t)$. We can then introduce the stochastic work dW_t as a function of time associated with the movement of the system dX_t :

$$dW_t = F(X_t) \circ dX_t, \quad (21)$$

where the symbol \circ indicates that the stochastic integration is in the Stratonovich sense (23). We can also introduce a stochastic entropy Ψ_t ,

$$\Psi_t = -k_B \ln P(X_t, t). \quad (22)$$

Then Onsager’s thermodynamic force Π_t is in fact

$$\Pi_t = F(X_t) + T \nabla \Psi_t(X_t), \quad (23)$$

which is the mechanical force plus the entropic force. If $F(X_t)$ can be written as $-\nabla U(X_t)$, then $\Pi_t = -\nabla \{U(X_t) - k_B T \ln P(X_t, t)\}$. The term in the brackets is in fact the free energy. Hence, Onsager’s thermodynamic force is the gradient of the chemical potential (48, 49). Finally, we can introduce the stochastic dissipation dQ_t associated with the movement dX_t :

$$dQ_t = \Pi_t \circ dX_t. \quad (24)$$

The mean dissipation rate, $d\langle Q_t \rangle / dt$, is the *epr* in Equation 17, and the mean $d\langle W_t \rangle / dt$ is the *hdr* in Equation 17.

For a closed system, $G(x) = 0$ and $F(x) = -\nabla U(x)$. Then we have $dW_t = -dU(X_t)$, $\Psi_t = -k_B \ln P(X_t, t)$, $\Pi_t = -\nabla (U(X_t) - T \Psi(X_t)) = -\nabla \mu(X_t)$, and $dQ_t = -d\mu(X_t)$. In the equilibrium stationary state, we have $\langle dW(t) \rangle = 0$, the fluctuating $\Pi_t \equiv 0$, because $\mu(X_t) = \text{const.}$, and $dQ(t) \equiv 0$. More interestingly, for an open system driven at the boundary, there is no breakdown of detailed balance in the interior of the phase space; hence $G(x) = 0$. In this case, a nonconstant chemical

potential μ exists, and $\Pi_r \neq 0$. This formalism of a motor protein, known as rectified Brownian motion, can be found in Reference 43.

2.8. Mesoscopic Open–Nonlinear Chemical Reaction Systems

One of the essential differences between the open-system NESS of single macromolecules and the NESS of general nonlinear chemical reactions is the conservation of the number of molecules: precisely one in the former and a grand canonical ensemble in the latter. In other words, fluctuations in a single-molecule NESS are among the different states of the molecule, whereas fluctuations in nonlinear reactions are the number of molecules in the system. In recent years, through Gillespie's (32) work, the CME formalism for nonlinear–chemical reaction systems (50) has become widely appreciated. This approach to mesoscopic systems plays the same role as the law of mass action for macroscopic systems. It enables us to write mathematical equations for the stochastic dynamics of mesoscopic–chemical reaction systems. In fact, with an increasing system's size and number of molecules, we can show mathematically that the solution to a stochastic CME approaches that of the differential equations based on the law of mass action (51). Therefore, we should consider the CME approach a more complete mathematical theory of chemical kinetics. In addition to providing fluctuations, the theory also naturally encompasses a statistical thermodynamics.

To establish the CME as the mathematical framework for chemical reaction systems, we need to integrate the well-known chemical oscillations into the theory. Using the CME approach, we have recently studied nonlinear chemical oscillations in mesoscopic systems that exhibit both stochastic fluctuations and temporal complexity (34). The distinction between these two types of dynamics can be easily understood in terms of Equation 20, in which the first term on the right-hand side represents diffusion, and the second term represents drift. The diffusion term generates stochastic fluctuations. The drift term, however, contains deterministic, nonlinear dynamics. It can generate oscillatory or even chaotic temporal dynamics. The diffusion term diminishes with an increasing system's size and number of molecules, and in certain situations, pumping energy into the system has a similar effect. From this observation, we have concluded that in a driven open system, large temporal variations can be in fact biologically functional rather than mere stochastic fluctuations (34, 52). Energy derived from phosphorylation can suppress the stochastic fluctuations while promoting temporal complexity, making a cell behave more like a macroscopic machine. (See Reference 13 for more discussion.)

3. PHOSPHORYLATION-DEPHOSPHORYLATION CYCLE AND A NEW KIND OF COOPERATIVITY

The switching of enzymes and proteins between phosphorylated and dephosphorylated states is a universal biochemical process inside living cells (53, 54). But why has protein phosphorylation evolved to be the ubiquitous mechanism for regulating enzyme activities in biochemistry? Our current hypothesis is that the free energy derived from ATP hydrolysis is used to ensure the proper function of biochemical signaling.

Phosphorylation-dephosphorylation switch:

the biological activity of a protein is turned on by its phosphorylation and turned off by dephosphorylation

PdPC: phosphorylation-dephosphorylation cycle

This chemical energy is necessary for overcoming intrinsic biochemical noise from thermal agitations, small copy numbers, and limited affinities, guaranteeing precise and robust cell development and functions.

3.1. Phosphorylation-Dephosphorylation Cycle Kinetics as a Cellular Switch

Because of the central importance of the phosphorylation-dephosphorylation cycle (PdPC), I begin with a simple kinetic model for such a system. **Figure 3a** shows a biochemical switch with E and E^* being the inactive and active forms of an enzyme, respectively. A complete cycle, $E \rightarrow E^* \rightarrow E$, is accompanied by one ATP hydrolysis. It is easy to show that the equilibrium constant for the ATP hydrolysis is

$$\frac{[ADP]^{eq}[P_i]^{eq}}{[ATP]^{eq}} = \frac{[ADP]^{eq}[E^*]^{eq}}{[ATP]^{eq}[E]^{eq}} \frac{[P_i]^{eq}[E]^{eq}}{[E^*]^{eq}} = \frac{k_1^o k_2}{k_{-1}^o k_{-2}^o}. \quad (25)$$

Hence, for given ATP, ADP, and P_i concentrations, the chemical energy available for one ATP hydrolysis is

$$\Delta\mu_{DT} = k_B T \ln \frac{k_1^o k_2 [ATP]}{k_{-1}^o k_{-2}^o [ADP][P_i]} = k_B T \ln \frac{k_1 k_2}{k_{-1} k_{-2}}, \quad (26)$$

where $k_1 = k_1^o [ATP]$, $k_{-1} = k_{-1}^o [ADP]$, and $k_{-2} = k_{-2}^o [P_i]$ are pseudo-first-order rate constants.

The kinetic equation is simple. We note that $E_T = [E] + [E^*]$, the total amount of enzyme molecules, is constant:

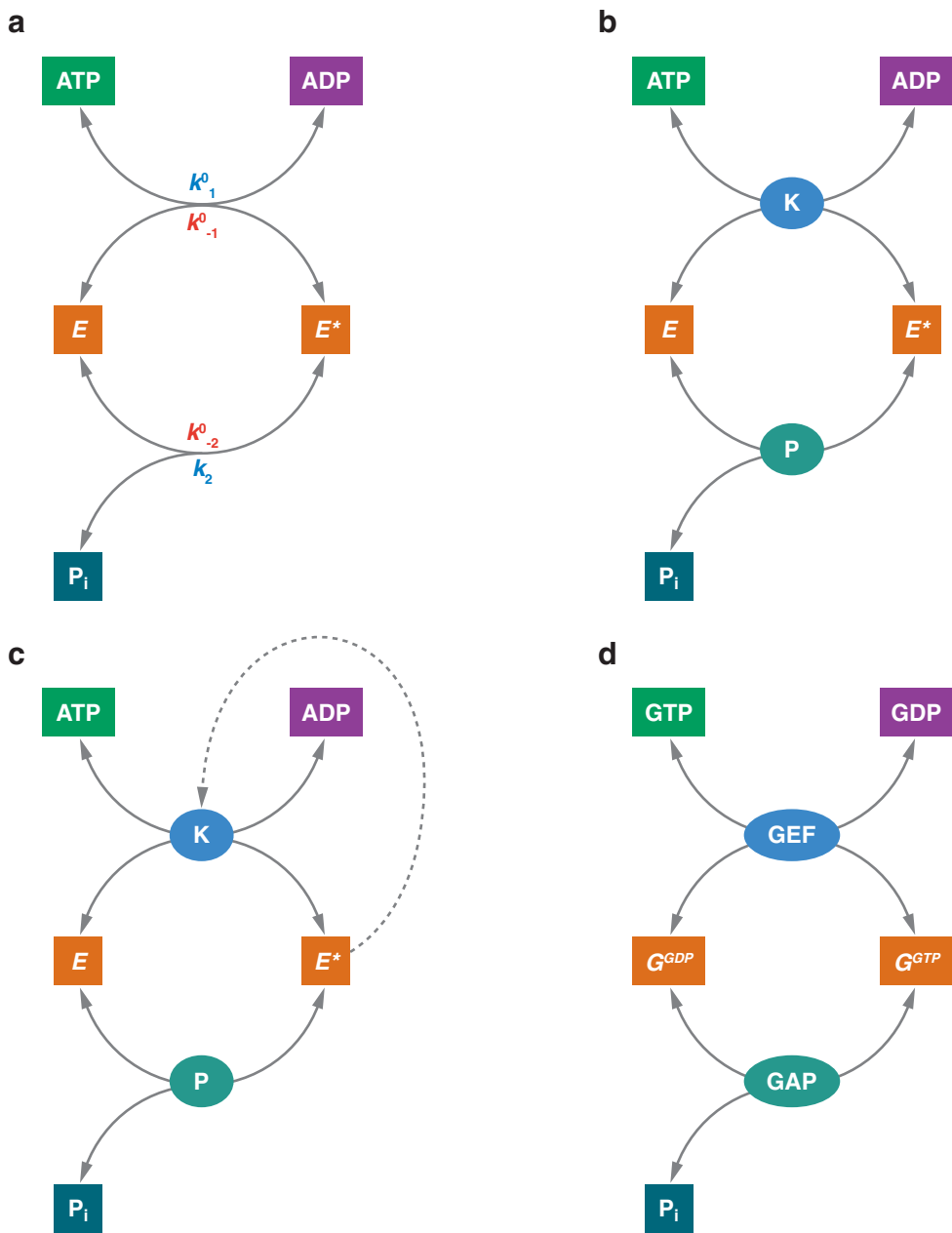
$$\frac{d[E]}{dt} = -\frac{d[E^*]}{dt} = -\mathcal{J}_1 + \mathcal{J}_2, \quad (27)$$

with

$$\mathcal{J}_1 = k_1 (E_T - [E^*]) - k_{-1} [E^*], \mathcal{J}_2 = k_2 [E^*] - k_{-2} (E_T - [E^*]). \quad (28)$$

Figure 3

Various kinetic models for cellular biochemical switches. For clarity, reversible reactions are represented by \leftrightarrow rather than \rightleftharpoons . (a) Simplest, nonenzymatic phosphorylation-dephosphorylation cycle (PdPC) with E and E^* as the nonphosphorylated and phosphorylated forms of a protein, respectively. k_1^o and k_{-1}^o are rate constants for phosphorylation and its reverse reaction, respectively. k_2 and k_{-2}^o are for dephosphorylation and its reverse reaction, respectively. k_1^o , k_{-1}^o , and k_{-2}^o are second-order rate constants. (b) PdPC with kinase and phosphatase as enzymes. If both enzymes are highly saturated, ultrasensitivity arises (55, 56). (c) If E^* has a positive feedback control for the kinase activity (*dashed line*), bistability can occur (7, 57). (d) The kinetics scheme of a guanosine triphosphatase (GTPase) cycle, another key cellular signal-transduction module (58). G^{GDP} and G^{GTP} are the GDP- and GTP-bound GTPase. The reactions are catalyzed by two enzymes: guanine-nucleotide exchange factor (GEF) and GTPase activation protein (GAP). The GTPase cycle is remarkably similar to the PdPC in *b*.



The fraction of the enzyme in the active form in steady state is

$$f = \frac{[E^*]}{[E] + [E^*]} = \frac{\theta + \mu}{1 + \theta + \mu + \theta/(\gamma\mu)}, \quad (29)$$

where $\theta = k_1/k_2$, $\mu = k_{-2}/k_2$, and $\gamma = k_1k_2/(k_{-1}k_{-2}) = \exp(\Delta\mu_{DT}/k_B T)$. We can consider θ as the controlling parameter for the switch. If there is no energy available, $\gamma = 1$. Then, $f = \mu/(1 + \mu)$ in Equation 29, which is in fact independent of θ . In other words, if there is no energy, there is no switch. If μ is small and there is sufficient free energy ($\gamma \gg 1/\mu$), then

$$f \approx \frac{\theta}{1 + \theta}. \quad (30)$$

There is a transition from $f = 0$ to $f = 1$ when θ changes from 0 to ∞ . We can find the sharpness of the transition in Equation 29 (**Figure 4a**) as a function of θ , and we can define this sharpness as

$$\left(\frac{df}{d \ln \theta} \right)_{f=\frac{1}{2}} = \frac{(1 - \mu)(\mu\gamma - 1)}{4\mu(\gamma - 1)}. \quad (31)$$

The Hill coefficient of the transition, a widely used concept borrowed from allosteric cooperativity, is

$$n_b = 4 \left(\frac{df}{d \ln \theta} \right)_{f=\frac{1}{2}}. \quad (32)$$

The bottom curve in **Figure 5** shows how n_b increases with γ , with $\mu = 0.001$. For Equation 30, $n_b = 1$. The sharper the transition (i.e., the greater n_b), the greater the sensitivity of the switch to small changes in the controlling parameter.

3.2. Kinase, Phosphatase, and Ultrasensitivity

Can a biochemical switch undergo a transition sharper than $n_b = 1$? Based on the seminal theory of Goldbeter & Koshland (55), we recently developed a kinetic model for reversible PdPC with Michaelis-Menten kinetics for kinase and phosphatase. The fraction of enzyme in the active form in steady state, f , satisfies the equation (56)

$$\theta \equiv \frac{K_2 V_1}{K_1 V_2} = \frac{\mu\gamma[\mu - (\mu + 1)f][K_1 + (1 - f)E_T]K_2}{[(\mu\gamma + 1)f - \mu\gamma]K_1(fE_T + K_2)}, \quad (33)$$

in which E_T is the total enzyme concentration, $E_T = [E] + [E^*]$, and K_1 , V_1 and K_2 , V_2 are the Michaelis constants and the maximal velocity of kinase and phosphatase, respectively. The ratio of the rate of the kinase to that of the phosphatase, θ , is again the controlling parameter, and μ and γ have the same meaning as above. If the total enzyme concentration, E_T , is sufficiently lower than K_1 and K_2 , then there is no kinase and phosphatase saturation. In that case, Equation 33 is reduced to $\theta = \mu\gamma[\mu - (\mu + 1)f]/[(\mu\gamma + 1)f - \mu\gamma]$, which agrees with Equation 29.

The Hill coefficient for Equation 33 is

$$n_b = \left[\frac{\mu(\gamma - 1)}{(1 - \mu)(\mu\gamma - 1)} - \frac{E_T(E_T + K_1 + K_2)}{(E_T + 2K_1)(E_T + 2K_2)} \right]^{-1}. \quad (34)$$

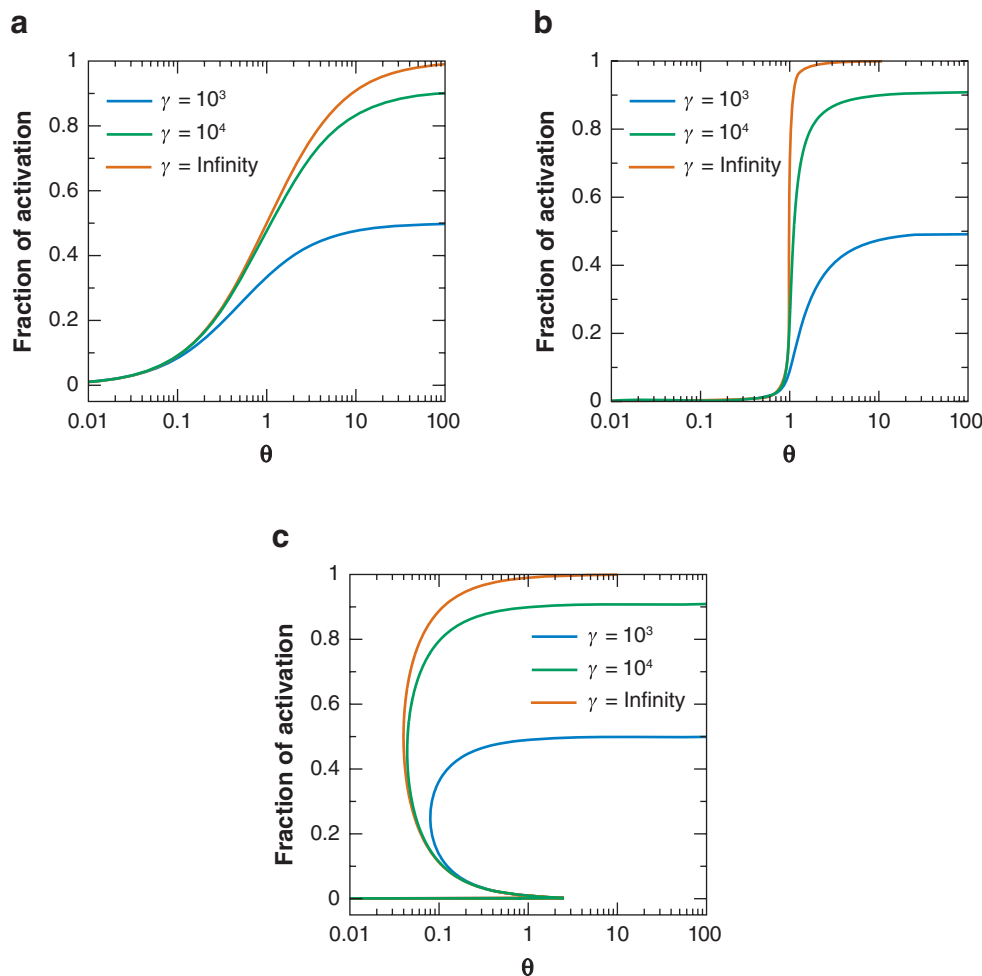


Figure 4

The phosphorylation-dephosphorylation cycle as a biochemical switch (*a*), with ultrasensitivity (*b*), and with nonlinear feedback (*c*), at different levels of available free energy. (*a*) Simple reaction kinetics in **Figure 3a**. Curves are according to Equation 29 with $\mu = 0.001$. (*b*) Kinase- and phosphatase-catalyzed reactions in **Figure 3b** with saturation. Curves are according to Equation 33 with $\mu = 0.001$, $K_1/E_T = K_2/E_T = 0.01$. The curves show a sharp transition. (*c*) Kinetics with feedback (**Figure 3c**). Curves are according to Equation 38 with $\mu = 0.001$, $E_T = 10$. These curves exhibit bistability.

Again, if $K_1, K_2 \gg E_T$, then this agrees with Equation 32. However, if $K_1, K_2 \ll E_T$, this is the condition for both kinase- and phosphatase-catalyzed reactions to be zeroth order. Then (56)

$$n_b = \left[\frac{1 - 2\mu + \mu^2\gamma}{(1 - \mu)(\mu\gamma - 1)} + \frac{K_1 + K_2}{E_T} \right]^{-1} \approx \left[\mu + \frac{1}{\mu\gamma} + \frac{K_1 + K_2}{E_T} \right]^{-1}, \quad (35)$$

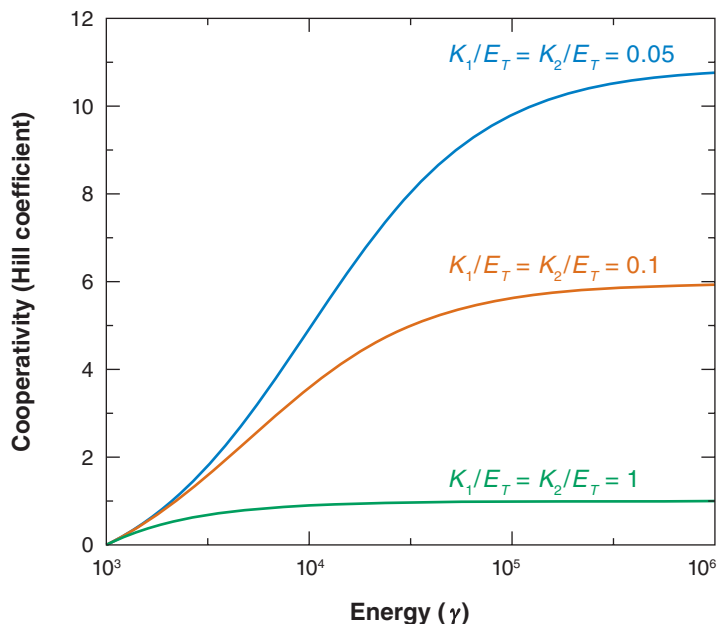


Figure 5

Hill coefficient in the phosphorylation-dephosphorylation cycle (PdPC) with ultrasensitivity. When $K_1/E_T = K_2/E_T = 1$, the curve is indistinguishable from Equation 32. With increasing saturation for both kinase and phosphatase, n_b increases. The computation is based on Equation 34 with $\mu = 0.001$. For $K_1/E_T = K_2/E_T = 0.01$, n_b approaches 48 (data not shown).

when $\mu \ll 1$ and $\mu\gamma \gg 1$. **Figure 4b** shows the ultrasensitive activation with different amounts of available energy. **Figure 5** shows how the Hill coefficient increases with increasing γ and decreasing $K_1/E_T, K_2/E_T$. The sensitivity of a switch in fact can be tuned by the amount of available energy, as well as the saturation level of the kinase and phosphatase. The latter mechanism is known as zeroth-order ultrasensitivity (55).

3.3. The New Concept of Temporal Cooperativity

The large Hill coefficient, n_b , in Equation 35 indicates high cooperativity. But what is the origin of this cooperativity? In the past, allosteric cooperativity in multisubunit proteins has been understood through subunit-subunit interaction. In the ultrasensitive zeroth-order PdPC, there is one kinase and one phosphatase, and there is no multisubunit protein involved.

To understand this new kind of cooperativity, let us first revisit some basic concepts. **Figure 6a** shows a sequential reaction involving four identical, independent proteins. Each protein has two conformational states (square and circle), with k_+ and k_- as the rate constants for the conformational transitions. The number of proteins in the circle

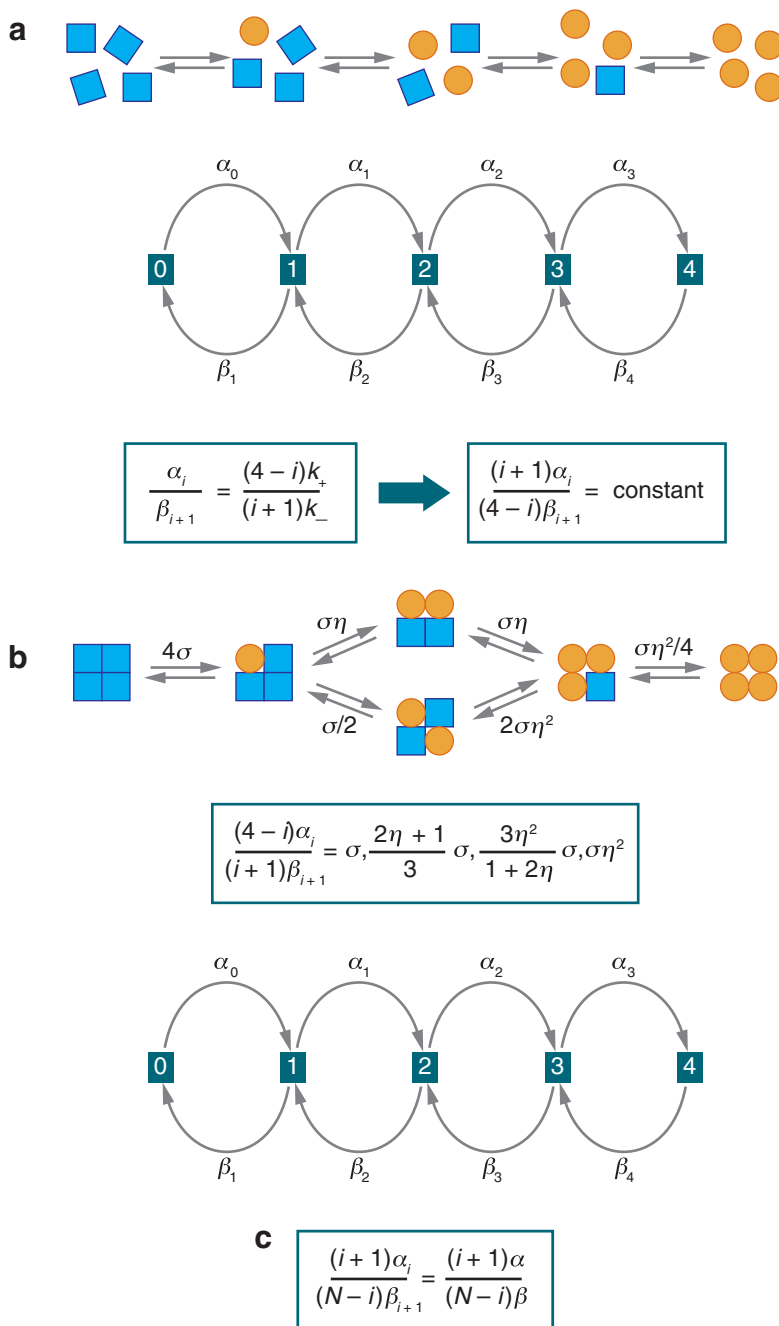
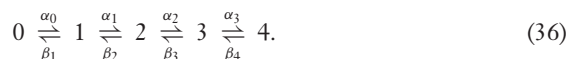


Figure 6

Noncooperative and cooperative systems. (a) In a noncooperative system with four subunits, each subunit undergoes a transition from a square to a circle. If all the subunits are independent, then the rate constants are $\alpha_i = (4-i)k_+$ and $\beta_i = ik_-$, with the integer in the kinetic scheme indicating the number of circles among the four subunits. The rule for a noncooperative system is $(i+1)\alpha_i/((4-i)\beta_{i+1}) = \text{constant}$. Deviation from this rule means cooperativity. (b) Koshland-Nemethy-Filmer-like cooperativity in which the subunits are interactive with their neighbors. The η parameter represents the interaction energy. Hence, if $\eta > 1$, there is a positive cooperativity, and if $\eta < 1$, there is a negative cooperativity. (c) In zeroth-order temporal cooperativity, there is no multisubunit interaction. Rather, the saturation of the enzyme reaction makes a zeroth-order transition between a square to a circle. Hence, all the α_i are the same, as are the β_i . There is a positive cooperativity according to the rule above.

Temporal cooperativity: a mechanism for sharp transition without allostery; the interactions between the players are in time, not in space

state characterizes the state of the system:



Then a simple combinatorial argument shows the apparent rate constants $\alpha_i = (4 - i)k_+$ and $\beta_i = ik_-$.

In general, for sequential kinetics of N identical, two-state proteins with apparent equilibrium constants α_i/β_{i+1} , the proteins are independent if the weighted ratio $(i + 1)\alpha_i/((N - i)\beta_{i+1})$ is independent of i . Conversely, if the weighted ratio increases with i , then the conformational changes are cooperative. The same principle applies to conformational changes in multisubunit proteins.

In the classic model of allosteric cooperativity, such as the one proposed by Koshland et al. (59) shown in **Figure 6b**, the rate constants α_i and β_i are functions of the free energy of the subunit-subunit interface interaction: $k_B T \ln \eta$. Hence the weighted ratio increases with i .

The meaning of zeroth order is precisely that $\alpha_i = \alpha$ and $\beta_i = \beta$; both are independent of the substrate concentration. Hence, the weighted ratio increases with i . This leads to cooperativity (i.e., ultrasensitivity). There is no direct interaction between the substrate enzymes. However, they all compete for the single kinase and phosphatase. Because this interaction is not through space, but instead is sequential in time, we refer to it as temporal cooperativity (56). (For more discussion on the comparison and relationship between allosteric cooperativity and the temporal cooperativity, see Reference 56.)

Conformational changes in enzymes can be regulated either by allosteric ligand binding or by covalent modification. Both lead to cooperative transitions. What is the difference between these two types of regulation and cooperativity? Fischer & Krebs (60), who discovered protein phosphorylation as a regulatory mechanism for enzyme activity, raised this question. In particular, Fischer et al. (53) ask (a) “[w]hy have organisms found it advantageous to develop separate mechanisms to control the activity of enzymes, namely, by noncovalent (allosteric) changes in structure mediated by appropriate effectors (binding), and by covalent modifications (via phosphorylation and ATP hydrolysis) of the proteins?” and (b) “[w]hy are these two mechanisms, i.e., noncovalent allosteric regulation and covalent modification via phosphorylation, usually superimposed on one another even though the changes in conformation resulting in either activation or inhibition are essentially the same?”

Our work (61, 62) has suggested that the essential difference between the allosteric mechanism and the hydrolysis cycle is that the former does not expend energy. Yet there are important tradeoffs associated with these two types of regulation. Regulation via the allosteric effect requires a sufficient quantity of regulator (proteins) to be present, in an amount approximately equal to that of the regulated enzyme. The PdPC approach requires only a relatively small amount of regulators (i.e., kinase and phosphatase) as catalysts. The costs of the two types of regulations are quite different. One requires a significant amount of regulator biosynthesis in advance. The other requires only a small amount of regulators for the hydrolysis reaction, but it consumes

energy during the regulation. In computer engineering terms, this is an issue of material cost-versus-energy utilization, an issue of overhead versus operational costs.

One can also analyze the temporal dynamics of the two types of regulation. Furthermore, if one investigates the nature of a signal in a biochemical regulation, then one finds one type of regulation can be more advantageous than another. For example, if regulatory molecules are in large concentrations (e.g., metabolites), then the allosteric interaction is natural. However, if regulatory information is represented by only a small number of copies of an active protein (such as in photon detection in the visual systems), the PdPC is more appropriate for the task.

3.4. Nonlinear Feedback and Bistability in Chemical Reaction Systems

Ferrell & Xiong (57) proposed a PdPC kinetic model with feedback, shown in **Figure 3c**. The dashed line in **Figure 3c** represents a positive feedback: E^* is an allosteric effector for the kinase (7).

For simplicity, we assume that both enzymes, the kinase and the phosphatase, are not saturated. An enzyme necessarily catalyzes both forward and backward reactions. Hence the \tilde{f} s in Equation 28 become

$$\begin{aligned}\tilde{f}_1 &= \{k_1(E_T - [E^*]) - k_{-1}[E^*]\} [K][E^*]^2, \\ \tilde{f}_2 &= \{k_2[E^*] - k_{-2}(E_T - [E^*])\} [P],\end{aligned}\quad (37)$$

where $[K]$ and $[P]$ are the concentrations of the kinase and phosphatase.

The fraction of the active form E^* in steady state, f , satisfies (7)

$$\theta = \frac{k_1[K]}{k_2[P]} = \frac{\mu\gamma[\mu - (\mu + 1)f]}{[(\mu\gamma + 1)f - \mu\gamma]E_T^2 f^2}.\quad (38)$$

Figure 4c shows a bistability in contrast to the sharp transition in **Figure 4b** owing to ultrasensitivity.

In a closed system with $\gamma = 1$, the bistability disappears. Equation 38 shows that there is a unique equilibrium, $(\mu + 1)f - \mu = 0$, the same as that of Equations 29 and 33. Mathematically, the bistable region moves to the negative part of θ : $\theta = -\mu/(E_T f)^2$, which has no physical meaning (7).

In an open system with $\gamma \gg 1$, there is a fundamental difference between the transitions in **Figures 4b,c**. At every given θ -value, the transition in **Figure 4b** has a unique steady state. The transition with increasing θ follows exactly the reverse path for decreasing θ . However in the transition in **Figure 4c**, when $0.03 \leq \theta \leq 3$, there are three coexisting steady states, with the one in the middle being unstable. The transition with increasing θ follows a path different from the one with decreasing θ . The difference between these two paths is called hysteresis, which is characteristic of nonlinear systems. (See Reference 7 for more discussion of the possible biological functions of such transitions.)

Microscopic conformation, mesoscopic concentration, and bistability. A system with multiple steady states in coexistence under identical conditions is called

multistable. The different steady states of the system are reached from different initial conditions. As discussed in Section 2.3, a chemical reaction system has a unique NESS with a probability distribution. Each local maximum in the mesoscopic concentration distribution, however, corresponds to a macroscopic steady state. The term bistability (see above) is used in this context. A mesoscopic system fluctuates between the two local maxima. However, the same chemical reaction system with macroscopic size remains in one of the local maxima because a transition between two maxima takes an astronomical time.

Bistability cannot occur in a closed macroscopic–chemical reaction system. With chemical detailed balance and assuming the law of mass action, one can show that a closed macroscopic system has a unique equilibrium steady state. Correspondingly, in a closed mesoscopic–chemical reaction system, the probability distribution of the concentrations (molecular numbers) has a single maximum.

This conclusion comes as a surprise because microscopic systems with multimima energy landscapes are common. The best example is the multiple conformational states of a protein (**Figure 7a**). However, there is an important distinction between mesoscopic systems in terms of molecular concentrations (numbers) and microscopic systems in terms of molecular conformation. To illustrate this, let us consider N copies of a protein molecule with the conformational distribution given in **Figure 7a** in a closed system. The equilibrium probability distribution for the number of molecules in the A -state is a binomial distribution with a single maximum at Np_A (**Figure 7b**).

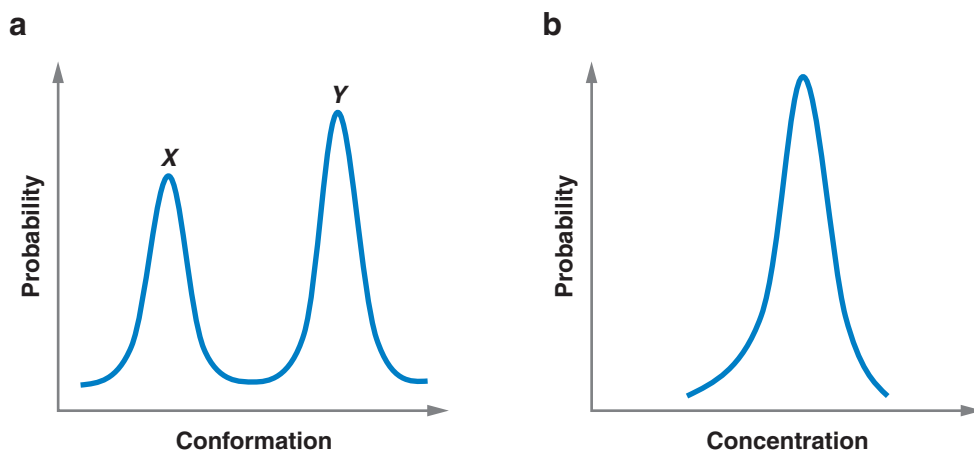


Figure 7

The difference between a microscopic probability distribution for molecular conformations in a closed system and a mesoscopic probability distribution for molecular concentrations in a closed system. (a) The equilibrium probabilities for conformations X and Y are dictated by their conformational energy (Boltzmann's law). A local minimum in energy function corresponds to a maximum in probability, and there can be many local energy minima. (b) The equilibrium probability distribution for the concentrations of molecular species, however, can only have a single maximum, corresponding to the unique macroscopic equilibrium concentrations.

4. SPECIFICITY AMPLIFICATION AND ITS THERMODYNAMIC LIMIT

In living cells, the specificity of biochemical recognition can be amplified and the intrinsic noise from nonspecific interference can be reduced at the expense of cellular free energy (6, 63, 64). This is the seminal idea in the theory of kinetic proofreading (6, 65). Although the molecular mechanisms of proofreading have been extensively elucidated in terms of the exonuclease activity of DNA polymerase (66) and for protein synthesis in terms of ribosome structure and kinetics (67), the role of free energy in biological error reduction (68) has not been as widely discussed.

Biological organisms, through evolution, have acquired a repertoire of mechanisms to counteract stochasticity (69, 70), thus improving the accuracy of their informational processing. However, this strategy relies on the energy resource available to the organism. In this section, I discuss open-system kinetic models that quantify the relationships between the level of available free energy and error reduction (71, 72).

Specificity is one of the most important concepts in molecular biology. The molecular basis of specificity is usually attributed to differences in affinity. In other words, an enzyme recognizes its natural substrate with higher affinity because there is a structural complementarity between the enzyme and its natural substrate.

However, this structural-based specificity is not everything. In the 1970s, Hopfield (6) and Ninio (65) independently discovered that the specificity can be regulated purely through chemical kinetic means without modifying molecular structures. This kinetic-proofreading mechanism has been shown to be responsible for the high fidelity in many important biological processes.

4.1. Kinetic Proofreading and Affinity Ratio

The theory of kinetic proofreading was developed to explain the high fidelity in biosynthetic processes (6, 65). The idea and the mathematical model, however, have much broader cellular applications and can be applied to other systems, such as receptor-ligand interactions coupled with hydrolysis (**Figure 8**) (58).

The equilibrium between the empty receptor R and the activated complex RL^* is $[RL^*]^{eq}/[R]^{eq} = k_{-3}^0[L]/k_3 = k_{-3}/k_3$. We again use the parameter $\gamma = (k_1 k_2 k_3)/(k_{-1} k_{-2} k_{-3})$ to represent the free energy from hydrolysis, $\Delta\mu_{DT} = k_B T \ln \gamma > 0$.

To study specificity, we consider two ligands L and L' at equal concentration. L and L' are structurally related, so they have the same k_1 , k_2 , k_{-2} , and k_{-3} . However, their affinities with the receptor are different owing to

$$\frac{k'_{-1}}{k_{-1}} = \frac{k'_3}{k_3} = \sigma. \quad (39)$$

If the receptor has a higher affinity for L' than for L , then $\sigma < 1$. In a chemical equilibrium (i.e., $\gamma = 1$), we can quantify the ratio of the two affinities:

$$A_r = \frac{[RL^*]/([R][L])}{[RL'^*]/([R][L'])} = \frac{k_{-3}/k_3}{k_{-3}/k'_3} = \sigma. \quad (40)$$

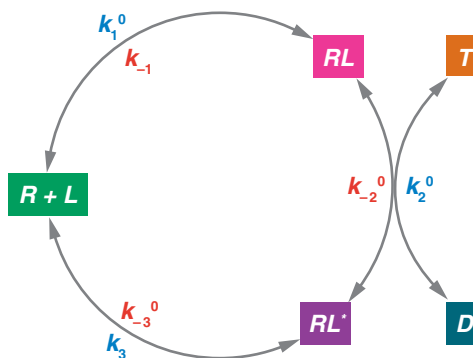


Figure 8

A three-state kinetic model of receptor-ligand binding coupled with a hydrolysis reaction. The biochemical literature often refers to RL^* as an activated complex with crucial biological activity. Rate constants with positive and negative subscripts are for clockwise and counterclockwise directions, respectively. k_1^0 , k_2^0 , k_{-2}^0 , and k_{-3}^0 are second-order rate constants. $k_1 = k_1^0[L]$, $k_2 = k_2^0[T]$, $k_{-2} = k_{-2}^0[D]$, and $k_{-3} = k_{-3}^0[L]$ are pseudo-first-order rate constants. Because the concentrations of T and D are not at equilibrium in living cells, $k_1 k_2 k_3 / (k_{-1} k_{-2} k_{-3}) = \gamma > 1$.

The affinity ratio A_r represents the relative probability of activation owing to non-specific binding, and $1/A_r$ represents the specificity of ligand L' with respect to L . The smaller A_r is, the greater the specificity.

In living cells, $[RL^*]$ and $[R]$ are not at their equilibrium owing to their coupling to the hydrolysis reaction in **Figure 8** ($RL + T \rightleftharpoons RL^* + D$). The affinity ratio therefore depends on how much energy is available; in other words, A_r is a function of γ (72):

$$A_r(\gamma) = \sigma \frac{(k_1 k_2 + k_2 k_{-3} + k_{-1} k_{-3}) \left(k_2 k_{-3} + \sigma k_{-1} k_{-3} + \frac{k_1 k_2}{\gamma} \right)}{\left(k_2 k_{-3} + k_{-1} k_{-3} + \frac{k_1 k_2}{\gamma} \right) (k_1 k_2 + k_2 k_{-3} + \sigma k_{-1} k_{-3})}. \quad (41)$$

We see that when $\gamma = 1$, $A_r = \sigma$. Given γ and σ , we can show that Equation 41 has a minimum

$$A_{r, \min}(\gamma) = \sigma \left(\frac{1 + \sqrt{\gamma \sigma}}{\sqrt{\gamma} + \sqrt{\sigma}} \right)^2, \quad (42)$$

when

$$k_{-1} \gg k_2, \quad k_1 \gg k_{-3}, \quad k_1 k_2 > \sigma k_{-1} k_{-3}, \quad k_3 > k_{-2}. \quad (43)$$

Inequalities $k_{-1} \gg k_2$ and $k_1 \gg k_{-3}$ imply that step 1 in **Figure 8** is in rapid equilibrium for a maximal specificity.

Figure 9 shows $A_{r, \min}$ as function of γ as given by Equation 42. When γ goes to infinity (i.e., there is a sufficient amount of energy available), $A_{r, \min}$ approaches σ^2 . This is the celebrated result of References 6 and 65. Equation 42 provides the best scenario, under the constraint of finite γ and the kinetic scheme in **Figure 8**, for specificity amplification, which can be defined as σ/A_r .

For kinetic schemes more complex than the one shown in **Figure 8**, we can achieve greater specificity amplification (72). Kinetic proofreading is not just the

Specificity amplification: specificity determined by molecular structures and equilibrium affinities can be increased in a living cell

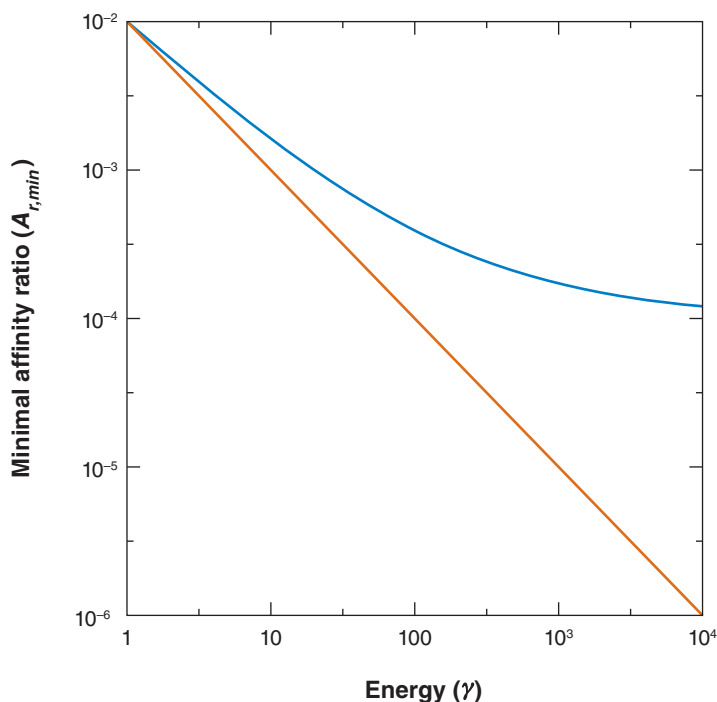


Figure 9

The orange line is the estimated absolute lower bound, based on thermodynamics, for the affinity ratio A_r with given energy γ . The blue line is the minimal affinity ratio for three-state Hopfield models, according to Equation 42.

mechanism for correcting errors in DNA replications and protein biosynthesis. It is a chemical kinetic mechanism of regulating specificity. The energy expenditure is a key ingredient of the mechanism. There is a unifying principle underlying the kinetic proofreading and the PdPC discussed in Section 3.

4.2. Thermodynamic Limit on Specificity Amplification with Finite Energy

Is it possible to obtain a lower bound for the affinity ratio with a given amount of energy γ independent of any kinetic scheme? In other words, is there a thermodynamic limit for specificity amplification irrespective of the detailed wiring diagram (i.e., **Figure 8**)?

We can write the competition between L and L' for R into a single biochemical reaction:



which has an equilibrium constant σ . With equal amounts of L and L' , the free energy difference between RL^* and RL'^* , $\Delta\mu^{eq}$, is zero at equilibrium:

$$\Delta\mu^{eq} = -k_B T \ln \sigma + k_B T \ln \frac{[RL^*]}{[RL'^*]} = 0. \quad (45)$$

In living cells, this reaction is coupled to an energy source with free energy $k_B T \ln \gamma$. The free energies in a reaction loop, including source and dissipation, satisfy

GTPase: guanosine triphosphatase

GTPase timer: the duration of a GTPase in its GTP-bound, active state used to time biochemical events

Kirchhoff's loop law (13, 73). Hence, the maximum contribution to the reaction, assuming no waste of energy in the coupling, is

$$\Delta\mu = -k_B T \ln \sigma + k_B T \ln \frac{[RL^*]}{[RL^*]} = -k_B T \ln \gamma. \quad (46)$$

This yields

$$\frac{[RL^*]}{[RL^*]} = \frac{\sigma}{\gamma}. \quad (47)$$

The orange line in **Figure 9** shows this absolute lower bound for the affinity ratio irrespective of any kinetic models. Equation 47 was first discovered in Reference 71. (For more discussion, see Reference 72.)

The above thermodynamic argument can be generalized. For example, if a proofreading process involves enzyme movement against a force, such as an RNA polymerase in a biophysical experiment (74), then the available energy to the proofreading is less than γ . In this case, the theory predicts that the thermodynamic limit for affinity ratio is $\frac{\sigma}{\gamma} e^{w/k_B T}$, where w is the amount of mechanical work done against the force. There is a trade-off between biochemical specificity and mechanical work.

5. KINETIC TIMING: MOLECULAR TIMER AND ITS ACCURACY

Guanosine triphosphatase (GTPase), another key cellular signal-transduction module (58), has a remarkably similar network structure to the PdPC. To see this, we can compare **Figures 3b,d**. The biological function of a GTPase is to control biochemical activities, again through switching, but also to time the activation of biological events, such as the duration of signaling (75, 76).

The lifetime T of a protein conformational state determines the duration of an activation process. For a single molecule, T is stochastic. In fact, if the biologically active state is indeed a single conformational state, then the duration of the activation is exponentially distributed: $\lambda e^{-\lambda t}$, where $\langle T \rangle = 1/\lambda$ is the mean time. An exponential distribution has a relative variance of

$$\frac{\text{Var}[T]}{\langle T \rangle^2} = 1. \quad (48)$$

That is, the distribution is broad. More importantly, the distribution is monotonic, and it does not peak at $\langle T \rangle$.

If the biologically active state consists of two conformational states (**Figure 10**), then the probability distribution for the lifetime of $B = B_1 \cup B_2$ has two exponential terms:

$$f_T(t) = a\lambda_1 e^{-\lambda_1 t} + (1-a)\lambda_2 e^{-\lambda_2 t}. \quad (49)$$

If the biochemical reactions are in a closed system that satisfies detailed balance, then we can show that $0 \leq a \leq 1$. That means the distribution is again monotonically decreasing, and the relative variance is now greater than 1 (76a):

$$\frac{\text{Var}[T]}{\langle T \rangle^2} = 2 \frac{a\lambda_2^2 + (1-a)\lambda_1^2}{(a\lambda_2 + (1-a)\lambda_1)^2} - 1 \geq 1. \quad (50)$$

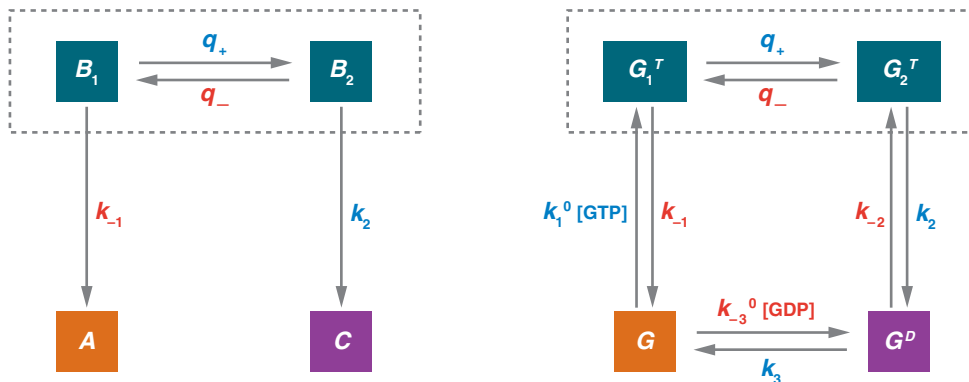


Figure 10

Kinetic schemes for a biomolecular conformation-based timer. (a) The biologically active state of a signaling molecule is B , which consists of B_1 and B_2 . The duration of the molecule in the B state is directly related to the integrated activity of the molecule. In a closed system with detailed balance, the probabilities of the molecule entering the B state through B_1 and B_2 are proportional to $k_{-1} P_{B_1}^{eq}$ and $k_2 P_{B_2}^{eq}$, respectively. (b) A model of GTPase systems in a living cell. G , $G_{1,2}^T$, and G^D are empty, GTP-bound, and GDP-bound GTPase, respectively. The existence of two conformations for the GTP-bound state has been suggested for N-ras P21 GTPase (77, 78). We introduce pseudo-first-order rate constants $k_1 = k_1^0 [GTP]$ and $k_{-3} = k_{-3}^0 [GDP]$. In the open system, the GTPase enters G^T dominantly through G_1^T .

As a timer, this is even less accurate than a single exponential. This result is general: In a closed system, any timer based on the lifetime of a group of conformational states can be no more accurate than one based on a single state.

The positive a and $(1 - a)$ in Equation 49 lead to a breakdown of causality. As an example, observer John starts a timer following Equation 49 at time zero. John finds a mean time of $a/\lambda_1 + (1 - a)/\lambda_2$. However, if observer Jean joins John at time $\tau > 0$ and records the same timer but starting only at τ , Jean has a mean time of

$$\frac{ae^{-\lambda_1\tau}/\lambda_1 + (1-a)e^{-\lambda_2\tau}/\lambda_2}{ae^{-\lambda_1\tau} + (1-a)e^{-\lambda_2\tau}} \geq \frac{a}{\lambda_1} + \frac{1-a}{\lambda_2}. \quad (51)$$

This paradox indicates that molecular timing based on conformational states cannot be carried out without ambiguity in a closed system. In other words, if there is no energy, there is no timer.

6. CONCLUDING REMARKS

Analyzing biochemical reaction systems (such as signal transduction, gene regulation, and metabolic networks) in terms of energetics and thermodynamics is a natural extension of physical chemistry. But what is the importance of such an endeavor to biology? One of the central questions in cellular biology is how a tiny cell (a mesoscopic collection of fluctuating molecules and noisy reactions driven by thermal energy) can accomplish the precisely programmed instructions in its genes. The more we understand biological functions in terms of their molecular processes, the more

surprised we are by the seeming impossibility of such small chemical systems behaving like macroscopic machines.

A cell is indeed a biochemical machine that exhibits complex temporal behavior, but the temporal dynamics cannot be dominantly stochastic. Free energy inputs assure that the dynamics of the biochemical reaction system is, as required by evolution, not dictated by thermal noise. As discussed above, important biochemical reactions inside living cells execute robust temporal dynamics, by carrying out a wide range of functions, such as toggle switching through the PdPC, decision making through specificity amplification, and counting time. All in all, information processing in cellular biology requires free-energy expenditure for its accuracy. It is thus natural that evolution has chosen the energy-rich phosphorylation reactions as the ubiquitous mechanism for signal transduction inside cells.

SUMMARY POINTS

1. Far-from-equilibrium thermodynamics of open chemical systems is the physicochemical foundation of the dynamics and functions of biochemical processes inside living cells.
2. No matter how complex a chemical reaction system is, without free energy input and dissipation, many cellular networks cannot function.
3. In biochemical reactions involved in signaling, high-grade chemical energy is reduced to low-grade heat; the energy involved in processing information must be explained in terms of entropy production.
4. Free energy derived from cycles of phosphorylation and dephosphorylation of proteins can be used to improve specificity in biomolecular recognition and robustness in cell development.
5. Evolution has chosen the energy-rich phosphorylation reactions as the ubiquitous mechanism for signal transduction inside cells.
6. Free energy dissipation (i.e., negative entropy) makes the cell—a mesoscopic-biochemical reaction system that consists of a collection of thermally driven fluctuating macromolecules—a genetically programmed chemical machine.

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