Enzyme kinetics are cyclic. We study a Markov renewal process model of single-enzyme turnover in nonequilibrium steady-state (NESS) with sustained concentrations for substrates and products. We show that the forward and backward cycle times have identical non-exponential distributions: \( \Theta_+(t) = \Theta_-(t) \). This equation generalizes the Haldane relation in reversible enzyme kinetics. In terms of the probabilities for the forward \( p_+ \) and backward \( p_- \) cycles, \( k_B T \ln(p_+/p_-) \) is shown to be the chemical driving force of the NESS, \( \Delta \mu \). More interestingly, the moment generating function of the stochastic number of substrate cycle \( \nu(t) \), \( \langle e^{-\lambda \nu(t)} \rangle \) follows the fluctuation theorem in the form of Kurchan-Lebowitz-Spohn-type symmetry. When \( \lambda = \Delta \mu/k_B T \), we obtain the Jarzynski-Hatano-Sasa-type equality: \( \langle e^{-\nu(t)\Delta \mu/k_B T} \rangle \equiv 1 \) for all \( t \), where \( \nu \Delta \mu \) is the fluctuating chemical work done for sustaining the NESS. This theory suggests possible methods to experimentally determine the nonequilibrium driving force \( \text{in situ} \) from turnover data via single-molecule enzymology.

Keywords: fluctuation theorem, Jarzynski’s equality, nonequilibrium steady-state, single-molecule enzymology

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Most biochemical reactions in a living cell have nonzero flux \( J \) and nonzero chemical driving force \( \Delta \mu \). The nonequilibrium state of such a reaction is sustained by continuous material and energy exchange with and heat dissipation into its environment [1]. To understand the state of a biochemical network in an open environment, hence, it is necessary to be able to experimentally measure both \( J \) and \( \Delta \mu \) \( \text{in situ} \). A large literature exist on measuring \( J \), but none exists on directly measuring \( \Delta \mu \). One could in principle compute \( \Delta \mu \) from \( \text{in situ} \) measurements of the concentrations of the substrate and product of a reaction if its equilibrium constant is known [2]. Alternatively, one should be able to obtain \( \Delta \mu \) from fluctuating cycle kinetics of a single enzyme directly. This possibility has been recently investigated in term of stochastic simulations [3]. Here we exam this idea through a novel analytical model.

Enzyme kinetics are complex mainly due to the many possible intermediates in the form of enzyme-substrate complexes. Recent laboratory measurements with high resolution at the single-molecule level give the waiting time distributions for enzyme cycles [4]. This motived the present Markov renewal process (MRP) model, also known as extended kinetics model in the theory of motor proteins [5]. In terms of the MRP, the kinetics of a single enzyme becomes a stochastic sequences of forward and backward cycles as function of time. We shall denote the number of forward and backward cycles by \( \nu_+(t) \) and \( \nu_-(t) \), as shown in Fig. 1.

It is obvious that the cycle time distributions give information on the kinetics. In this letter we show that the key nonequilibrium thermodynamic quantity, \( \Delta \mu \), can be obtained from stochastic data on single-enzyme cycle \( \nu(t) \equiv \nu_+(t) - \nu_-(t) \) via two novel equalities

\[
\Delta \mu = k_B T \ln \left( \frac{\langle \nu_+(t) \rangle}{\langle \nu_-(t) \rangle} \right); \quad (1)
\]

\[
\langle e^{-\nu(t)\Delta \mu/k_B T} \rangle = 1, \quad \forall t, \quad (2)
\]
where \( \langle \cdots \rangle \) is ensemble average for repeated measurement of \( \nu(t) \) in steady-state. Eq. 1 generalizes a result well known for one-step chemical reactions 1, 3, 4. Eq. 2 is a version of the fluctuation theorem (FT) in nonequilibrium statistical mechanics. The FT for the probability distribution of entropy production of a nonequilibrium steady-state (NESS) was first discovered in deterministic dynamical systems 5. Kurchan, Lebowitz and Spohn (KLS) introduced a parallel theory in terms of stochastic dynamics 5 which is more appropriate for single enzyme experiments 4, 8, 9, 10. It was shown that the generating function, i.e., an exponential average, of a work functional \( W(t) \) possesses a certain symmetry in the limit of \( t \to \infty \). Crooks introduced a heat functional \( Q(t) \) and showed the similar symmetry is valid for all finite \( t \): 
\[
\lambda(t) = c_1 - \lambda(t) \quad \text{where} \quad c_1(t) = \langle e^{-\lambda(t) / k_BT} \rangle .
\]
Since \( Q(t) \) and \( W(t) \) differ by a stationary term while both increase without bound, Crooks’ result immediately yields that of KLS’s. The symmetry in the generating function implies FT for \( Q \) 12.

The symmetry implies that \( \ln \langle e^{-Q(t) / k_BT} \rangle = 0 \). This is analogous to the Jarzynski equality 13 which is surprising since \( \langle Q(t) \rangle = -\ln \langle e^{-Q(t) / k_BT} \rangle \) is the mean heat dissipated from the NESS which certainly \( \neq 0 \); it should always \( > 0 \). The Jarzynski equality provides the possibility to obtain a function of state such as free energy from an nonstationary heat functional \( Q(t) \) with finite \( t \). This was proposed and experimentally tested for mechanical work functional on single biological macromolecules such as RNA 14.

The difference between the FTs for \( W(t) \) in the limit of infinite \( t \) and for \( Q(t) \) with any finite \( t \) is crucial to real experiments. In heuristic thermodynamic terms, the work functional \( W(t) \) 5 is related to the \( \Delta \mu^* \) of a reaction and the heat functional \( Q(t) \) 11, to the \( \Delta \mu \). While the former is determined by the transition rate constants, hence experimentally accessible in short time, the latter depends on the stationary probability. For cyclic enzymatic turnovers, however, \( W = Q \). Hence, the FT associated with enzyme cycle kinetics is particularly simple, and experimentally accessible 5. Generalizing the Jarzynski equality to open-system, Hatano and Sasa’s equality for NESS 12 also suggested a possibility of computing chemical driving force for single-molecule chemical reactions in NESS, see 2, 17.

To show Eqs. 1 and 2, there are two strategies. One is based on traditional Markov models, i.e., master equations, for single enzyme kinetics. Then both equations can be show as consequences of the existing FTs 5, 11. An alternative, more insightful approach however, is to model the kinetics in terms of a MRP with cycle kinetics. In this new model, we shall show a suprising equality between the forward and backward cycle time distributions: 
\[
\Theta_+(\tau) = \Theta_-(\tau) .
\]
With this equality, Eq. 1 becomes obvious, and Eq. 2 can be shown in elementary terms, in the Eqs. 11, 12 below.

The equality \( \Theta_+(\tau) = \Theta_-(\tau) \) turns out to be a very important, unknown relation in enzyme kinetics. This is a key result of this work. It has to do with microscopic reversibility. There are experimental evidence for it, as well as theoretical models proving equal mean time \( \langle \Delta T_+ \rangle = \langle \Delta T_- \rangle \) 16, 17. We shall give a proof for the equal distribution with sequential enzyme kinetics. The proof for more general systems will be published elsewhere 15.

The Markov renewal process (MRP) model.— Detailed kinetic scheme of an enzyme catalyzed biochemical reaction \( A \rightleftharpoons B \) is usually very complex 19. But if one concerns only with the net number of steady-state turnovers from \( A \) to \( B \), \( \nu(t) \), it can be represented by a continuous-time, discrete-state one dimensional random walk with cumulative cycle time distribution functions \( \Theta_+(t) \) for the forward and the backward stochastic transition times \( \Delta T_+ \) and \( \Delta T_- \): \( \Theta_+(0) = 0, \Theta_+(\infty) = 1 \), and \( \Theta_+(t) \) are nondecreasing. This is a class of stochastic models known as MRP 20 which has wide applications in single-enzyme kinetics and motor protein stepping 5, 21. See Fig. 2 in which \( \nu_\pm = p_\pm \Theta_\pm(t) \), and \( p_+ + p_- = 1 \). \( p_+ \) (\( p_- \)) is the eventual probability of the enzyme binding \( A \) (\( B \)) and converting it to \( B \) (\( A \)). We shall also denote \( \nu(t) = \nu_\rightarrow(t) + \nu_\leftarrow(t) \).

We discover that a necessary condition for the Eqs. 1 and 2 is the cycle-time distributions for the forward and backward steps being equal: \( \Theta_+(t) = \Theta_-(t) \). We call this equality generalized Haldane equation 22.

![FIG. 2: (a) A schematic for an enzyme reaction converting substrate \( A \) to product \( B \). In a NESS, the concentrations for \( A \) and \( B \), \( c_A \) and \( c_B \), are controlled through feedback by an experimenter. The cumulative number of \( B \) taken out by the time \( t \) is denoted by \( \nu(t) \). \( -\infty < \nu(t) < \infty \). (b) The integer \( k \) be the successive increments of \( \nu(t) \) by a random walk with forward and backward time distributions \( \nu_\rightarrow(t) \) and \( \nu_\leftarrow(t) \).](image)
$w_+(t)$ is the joint probability for continuous $\Delta T$ and binary $\Delta \nu$:

$$w_+ = \text{Pr}(\Delta \nu = \pm 1, \Delta T \leq t), \quad (\ell \geq 1). \quad (3)$$

The equation $\Theta_+ = \Theta_0$ leads to $w_+(t) = p_+w(t)$. That is the random variables $\Delta \nu$ and $\Delta T$ are statistically independent!

$\Theta_+(t) = \Theta_0(t)$ and microscopic reversibility.— To show this equality for forward and backward cycles, we consider a sequential enzyme reaction as shown in Fig. 3a and a corresponding exit problem shown in 3b.

$$\frac{1}{w_1w_2...w_n} d^{m}w_{-n}(0) = \frac{1}{u_1u_2...u_n} d^{m}w_{+n}(0). \quad (4)$$

Since functions $w_+(t)$ and $w_-(t)$ are completely determined by these initial conditions which satisfy the linear algebraic system, we have

$$\frac{w_+(t)}{w_-(t)} = \prod_{\ell=1}^{n} \left( \frac{u_\ell}{w_\ell} \right) = e^{-\Delta \mu/k_BT}, \quad (5)$$

independent of $t$. That is $\Theta_- = \Theta_0(t)$.

The meaning of the equality now becomes clear: We recall that $u_1$ and $w_n$ are pseudo-first order rate constants: $u_1 = u_1^0c_A$ and $w_n = w_n^0c_B$. Then from Eq. 9 we have the KLS symmetry:

$$\frac{c_B}{c_A} = \frac{u_1^0u_2u_3...u_{n-1}u_n}{w_1w_2w_3...w_{n-1}w_n}, \quad (6)$$

that is $w_+(t) = w_-(t)$. Therefore, in a chemical equilibrium, not only on the average $w_+(\infty) = w_-(\infty)$, i.e., the forward flux equals the backward flux, but the detailed kinetics for the transition time distributions have to be equivalent: There is absolutely no statistical difference between the forward and backward reactions. In a NESS when $\Theta_0$ is not held true, $w_+(t) \neq w_-(t)$. But the difference is only in the total probability $p_+ = w_+(\infty)$ and $p_- = w_-(\infty)$, the distribution functions $\Theta_+(t) = \Theta_0(t)$ still hold true. This equality is essential to the KLS symmetry below. It is known that microscopic reversibility has to be satisfied even when a mesoscopic system is in a nonequilibrium steady-state.

**Kurchan-Lebowitz-Spohn (KLS) symmetry and FT.—** For $k$ successive number of renewal events (forward plus backward turnovers) within time $[0, t]$, let us denote $(\nu_k, T_k) = \sum_{\ell=1}^{k}(\Delta \nu_\ell, \Delta T_\ell)$. The moment generating function for $\nu(t)$ is

$$g_\lambda(t) = \langle e^{-\lambda \nu(t)} \rangle \quad (7)$$

$$= \sum_{n=-\infty}^{\infty} \sum_{k=0}^{\infty} \text{Pr}\{\nu_k = n, T_k \leq t, T_{k+1} > t\} \quad \text{asymptotically independent!}$$

**FIG. 3:** (a) A schematic for an enzyme reaction converting $A$ to $B$. The transition time distribution of a single enzyme converting an $A$ to $B$, $w_+(t)$, and converting a $B$ to $A$, $w_-(t)$, are intimately related to the exit problem shown in (b) in which $u_1$ and $w_n$ are pseudo-first order rate constants which depend on the concentrations of $A$ and $B$, respectively: $u_1 = u_1^0c_A$, $w_n = w_n^0c_B$. The scheme in (b) has been used to compute steady-state one-way flux in T. L. Hill’s theory on biochemical cycle kinetics.

$$\left\{ \sum_{k=0}^{\infty} \sum_{n=-k}^{\infty} e^{-\lambda n} \text{Pr}\{\nu_k = n\} \right\} \text{Pr}\{T_k \leq t, T_{k+1} > t\} \quad (8)$$

The Eq. 9 is obtained due to the independence between $\nu_k$ and $T_k$. Then from Eq. 9 we have the KLS symmetry

$$g_\lambda(t) = g_{\lambda^-}(t), \quad \forall t, \quad (10)$$

where $\lambda^* = \ln(p_+/p_-)$. Furthermore,

$$g_{\lambda^*}(t) \equiv \langle e^{-\nu(t)\Delta \mu/k_BT} \rangle = g_0(t) = 1, \quad (11)$$

if $\ln(p_+/p_-) = \Delta \mu/k_BT$ holds true. We recognize that $\nu(t)\Delta \mu$ is the external chemical work done to the system in NESS. Hence Eq. 11 is analogous to the Jarzynski equality for a cycle.

**Chemical driving force for NESS enzyme cycle.—** If we let the $t \rightarrow \infty$ in Eq. 5, we have $\ln(p_+/p_-) \equiv \lambda^* = \Delta \mu/k_BT$, which is needed in deriving Eq. 11. This generalizes the well-known result for single step chemical reactions to any complex enzyme reaction cycle.

We are now also in a position to show Eq. 10. The mean number of net turnovers can be computed from the $g_\lambda(t)$ given in Eq. 10:

$$\langle \nu(t) \rangle = \langle \nu_+(t) \rangle - \langle \nu_-(t) \rangle = -\left[ \frac{dg_\lambda(t)}{d\lambda} \right]_{\lambda=0} \quad (12)$$

$$= (p_+ - p_-) \sum_{k=0}^{\infty} k \text{Pr}\{T_k \leq t, T_{k+1} > t\} \quad (13)$$

$$= (p_+ - p_-) \times \text{mean # of cycles in time} \quad (14)$$
Therefore, \( \langle \nu(t) \rangle = \frac{\langle \nu_a(t) \rangle}{\langle \nu_0(t) \rangle} \). Furthermore, in the limit of large time, \( \langle \nu(t) \rangle \approx (p_+ - p_-) t / T_1 \), where \( T_1 = \int_0^\infty t dw(t) \) is the mean time for one cycle, forward or backward. When \( p_+ = p_- \), the steady-state flux \( J = \lim_{t \to \infty} \langle \nu(t) \rangle / t = 0 \) as expected. When \( p_+ > p_- \), \( J > 0 \).

**Summary.**— Studying enzyme-catalyzed biochemical reactions in situ requires methods for measuring \( \Delta \mu \), the NESS chemical driving force. Currently none exists. We propose obtaining \( \Delta \mu \) from stochastic cycle data of a single enzyme molecule, \( \nu(t) \), via (i) an equality similar to that of Jarzynski and Hatano-Sasa: \( e^{-\nu(t)}(\Delta \mu / k_B T) = 1 \); or simply (ii) \( k_B T \ln(\nu_+ / \nu_-) \). We developed a MRP model for the enzyme cycles with arbitrary complex mechanism, and have found an equality between the forward and backward cycle time distributions based on microscopic reversibility. This equality is a generalization of the what is known as Haldane relation for the reversible enzyme kinetics, and a recent result in \[17\].

The model enables us to establish a FT and above equalities (i) and (ii) for any \( t \). Noting that \( (1/t) \langle \nu(t) \rangle = J \), one thus obtains both the flux \( J \) and the driving force \( \Delta \mu \) for a reaction in NESS from the fluctuating \( \nu(t) \). The statistical accuracies associated with these measurements were discussed in \[8\].

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