Waiting Cycle Times and Generalized Haldane Equality in the Steady-State Cycle Kinetics of Single Enzymes

ARTICLE *in* THE JOURNAL OF PHYSICAL CHEMISTRY B · FEBRUARY 2008

Impact Factor: 3.3 · DOI: 10.1021/jp0729967 · Source: PubMed

1 AUTHOR:

Hao Ge
Peking University
43 PUBLICATIONS **503** CITATIONS

Waiting Cycle Times and Generalized Haldane Equality in the Steady-state Cycle Kinetics of Single Enzymes

Hao Ge*

April 15, 2009

Abstract

Enzyme kinetics are cyclic. A more realistic reversible three-step mechanism of the Michaelis-Menten kinetics is investigated in detail, and three kinds of waiting cycle times T, T_+, T_- are defined. It is shown that the mean waiting cycle times $\langle T\rangle$, $\langle T_+\rangle$, and $\langle T_-\rangle$ are the reciprocal of the steady-state cycle flux J^{ss}, the forward steady-state cycle flux J_+^{ss} and the backward steady-state cycle flux J_-^{ss} respectively. We also show that the distribution of T_+ conditioned on $T_+ < T_-$ is identical to the distribution of T_- conditioned on $T_- < T_+$, which is referred as generalized Haldane equality. Consequently, the mean waiting cycle time of T_+ conditioned on $T_+ < T_-$ ($\langle T_+|T_+ < T_-\rangle$) and the one of T_- conditioned on $T_- < T_+$ ($\langle T_-|T_- < T_+\rangle$) are both just the same as $\langle T\rangle$. In addition, the forward and backward stepping probabilities p^+, p^- are also defined and discussed, especially their relationship with the cycle fluxes and waiting cycle times. Furthermore, we extend the same results to the n-step cycle, and finally, experimental and theoretically based evidences are also included.

KEY WORDS: waiting cycle times; generalized Haldane equality; single-molecule experiment; nonequilibrium steady states; cycle flux; stepping probability

1 Introduction

Living cells function thermodynamically as open systems that are far from static thermal equilibrium, since cells must continually extract energy from their surroundings in order to sustain the characteristic features of life such as growth, cell division, intercellular communication, movement and responsiveness to their environment.

*School of Mathematical Sciences, Peking University, Beijing 100871, P.R.China; email: edmund_ge@tom.com

From the view of statistical physics, these stochastic models for systems biology exhibit nonequilibrium steady states (NESS) in which nonequilibrium circulations (cycle fluxes) necessarily emerge [36]. Hong Qian and his co-workers have recently discussed the relation between an NESS and traditional nonlinearly dynamics [38, 42, 37, 39].

The researches on irreversible systems far from equilibrium began with the works by Haken [16, 17] about laser and Prigogine, etc. [15, 34] about oscillations of chemical reactions. It is closely related to another concept of macroscopic irreversibility in nonequilibrium statistical physics. A macroscopic irreversible system in a steady state should have positive entropy production rate and should be in nonequilibrium.

T.L. Hill, etc. [18, 19, 20, 21] constructed a general mesoscopic model for the combination and transformation of biochemical polymers in vivid metabolic systems since 1966. Their results can be applied to explain the mechanism of muscle contraction and active transports [10].

Mathematical theory of nonequilibrium steady states and circulation (cycle fluxes) has been discussed for several decades since the original work [43, 44, 45, 46], in which Qian and co-workers developed the formulae for entropy production rate and circulation distribution of homogeneous Markov chains, Q-processes and diffusions, and moreover their relationship with reversibility. They concluded that the chain or process is reversible if and only if its entropy production vanishes, or iff there is no net cycle fluxes. Here, we recommend a recent book [26] for the systematic presentation of this theory.

Recently, we investigate the synchronized stochastic dynamics of a network model of yeast cell-cycle regulation [13], applying the mathematical theory of cycle fluxes (circulation) of Markov chains. In our model of yeast cell cycle, the trajectory concentrates around a main cycle with the dominant circulation, which we call stochastic limit cycle, that is the natural generalization of the deterministic limit cycle in the stochastic system.

Recent advances in single-molecule spectroscopy and manipulation have now made it possible to study enzyme kinetics at the level of single molecules, where the stochastic effects, termed as "dynamic disorder", are significant. Experimentalists can not only directly measure the distributions of molecular properties through single-molecule experiments rather than the ensemble average, but also apply the theory of stochastic processes to analyze the statistical properties of the stochastic trajectory [29, 49, 50, 51].

Xie, et.al [31, 32, 9, 28] observed that the mean waiting time is the same as the reciprocal of the Michaelis-Menten steady-state flux (i.e., the cycle flux in my language). But the model they built in their theoretical analysis is the simplest irreversible Michaelis-Menten mechanism, and the state space of their stochastic model (Markov chain) actually only contains two states (E and ES), which does not distinguish the two different pathways

from ES to E and is always in mathematical detailed balance rather than chemical detailed balance. That is just why they can only directly using the ordinary differential equations to get the explicit distribution function $f(t)$ of the waiting time, and avoid applying the strong Markov property, which is the basic method to compute mean waiting times in stochastic processes. So their method can not be generalized to more complicated cases, and generally speaking, the explicit distribution function $f(t)$ can rarely be obtained in such an analytic form.

In the present paper, a more realistic reversible three-step mechanism of the Michaelis-Menten kinetics is investigated in detail, and three kinds of waiting cycle times T, T_+, T_- are defined. It is shown that the mean waiting cycle times $\langle T\rangle$, $\langle T_+\rangle$, and $\langle T_-\rangle$ are the reciprocal of the steady-state cycle flux J^{ss}, the forward steady-state cycle flux J_+^{ss} and the backward steady-state cycle flux J_-^{ss} respectively.

We also show that the distribution of T_+ conditioned on $T_+ < T_-$ is identical to the distribution of T_- conditioned on $T_- < T_+$, which is referred as generalized Haldane equality [41]. This is a key result of this work. There is experimental evidence for it, as well as theoretical models proving equal mean time [3, 27, 28].

Consequently, the mean waiting cycle time of T_+ conditioned on $T_+ < T_-$ ($\langle T_+|T_+ < T_-\rangle$) and the one of T_- conditioned on $T_- < T_+$ ($\langle T_-|T_- < T_+\rangle$) are both just the same as $\langle T\rangle$. In addition, the forward and backward stepping probabilities p^+, p^- are also defined and discussed, especially their relationship with the cycle fluxes and waiting cycle times. Furthermore, we extend the same results to the n-step cycle, and finally, experimental and theoretically based evidences are also included.

2 Single enzyme kinetics: cycle flux and NESS

This subsection is just a brief introduction to our model and cycle fluxes, which is from Ref. [36].

We consider a more realistic three-step mechanism of the Michaelis-Menten kinetics in which the conversion of S into P in the catalytic site of the enzyme is represented as a process separate from release of P from the enzyme (Fig. 1(a)):

$$E + S \underset{k_{-1}}{\overset{k_1^0}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EP \underset{k_{-3}^0}{\overset{k_3}{\rightleftharpoons}} E + P. \tag{1}$$

If there is only one enzyme molecule, then from the enzyme perspective, the kinetics are stochastic and cyclic, as shown in Fig. 1 (b), with the pseudo-first-order rate constants $k_1 = k_1^0 c_S$ and $k_{-3} = k_{-3}^0 c_P$ where c_S and c_P are the sustained concentrations of substrate S and P in the steady state.

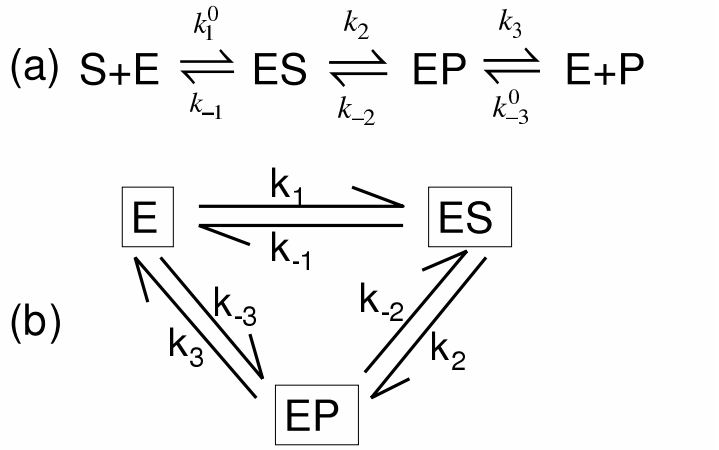

Figure 1: Kinetic scheme of a simple reversible enzyme reaction (a) in which k_1^0 and k_{-3}^0 are second-order rate constants. From the perspective of a single enzyme molecule, the reaction is unimolecular and cyclic (b). The pseudo-first-order rate constants $k_1 = k_1^0 c_S$ and $k_{-3} = k_{-3}^0 c_P$ where c_S and c_P are the concentrations of substrate S and P in the steady state.

At the chemical equilibrium, the concentrations of S and P satisfy $\frac{c_P}{c_S} = \frac{k_1^0 k_2 k_3}{k_{-1} k_{-2} k_{-3}^0}$, i.e.

$$\frac{k_1 k_2 k_3}{k_{-1} k_{-2} k_{-3}} = 1. \tag{2}$$

This is the "thermodynamic box" in elementary chemistry, also known as Wegscheider's relation and detailed balance. However, if the c_S and c_P are maintained at constant levels that are not at chemical equilibrium, as metabolite concentrations are in living cells, the the enzyme reaction is in an open system that approaches a NESS. This is the scenario in enzyme kinetics.

In this case,

$$\frac{k_1 k_2 k_3}{k_{-1} k_{-2} k_{-3}} = \gamma \neq 1, \tag{3}$$

and $\triangle\mu = k_B T \ln \gamma$ is well known as the cellular phosphorylation potential.

From the perspective of single enzyme molecule, the rate equation for the probabilities of the states is a master equation

$$\begin{aligned}
\frac{dP_E(t)}{dt} &= -(k_1+k_{-3})P_E(t)+k_{-1}P_{ES}(t)+k_3P_{EP}(t)\\
\frac{dP_{ES}(t)}{dt} &= k_1P_E(t)-(k_{-1}+k_2)P_{ES}(t)+k_{-2}P_{EP}(t)\\
\frac{dP_{EP}(t)}{dt} &= k_{-3}P_E(t)+k_2P_{ES}(t)-(k_{-2}+k_3)P_{EP}(t)
\end{aligned} \tag{4}$$

The steady-state probabilities for states E, ES and EP are easy to compute by setting the time derivative to zero and noting that $P_E+P_{ES}+P_{EP}=1$ for the total probability.

$$\begin{aligned}
P_E^{ss} &= \frac{k_2k_3+k_{-1}k_3+k_{-1}k_{-2}}{k_1k_2+k_2k_3+k_3k_1+k_{-1}k_{-3}+k_{-2}k_{-3}+k_{-1}k_{-2}+k_1k_{-2}+k_2k_{-3}+k_3k_{-1}},\\
P_{ES}^{ss} &= \frac{k_1k_3+k_{-2}k_{-3}+k_1k_{-2}}{k_1k_2+k_2k_3+k_3k_1+k_{-1}k_{-3}+k_{-2}k_{-3}+k_{-1}k_{-2}+k_1k_{-2}+k_2k_{-3}+k_3k_{-1}},\\
P_{EP}^{ss} &= \frac{k_1k_2+k_2k_{-3}+k_{-1}k_{-3}}{k_1k_2+k_2k_3+k_3k_1+k_{-1}k_{-3}+k_{-2}k_{-3}+k_{-1}k_{-2}+k_1k_{-2}+k_2k_{-3}+k_3k_{-1}}.
\end{aligned} \tag{5}$$

Then, the clockwise steady-state cycle flux in Fig. 1(b), which is precisely the enzyme turnover rate of $S\to P$ in Fig. 1(a), $J^{ss}=P_E^{ss}k_1-P_{ES}^{ss}k_{-1}=P_{ES}^{ss}k_2-P_{EP}^{ss}k_{-2}=P_{EP}^{ss}k_3-P_E^{ss}k_{-3}$, which follows

$$J^{ss}=\frac{k_1k_2k_3-k_{-1}k_{-2}k_{-3}}{k_1k_2+k_2k_3+k_3k_1+k_{-1}k_{-3}+k_{-2}k_{-3}+k_{-1}k_{-2}+k_1k_{-2}+k_2k_{-3}+k_3k_{-1}}=J_+^{ss}-J_-^{ss}, \tag{6}$$

where

$$J_+^{ss}=\frac{k_1k_2k_3}{k_1k_2+k_2k_3+k_3k_1+k_{-1}k_{-3}+k_{-2}k_{-3}+k_{-1}k_{-2}+k_1k_{-2}+k_2k_{-3}+k_3k_{-1}},$$

is the forward cycle flux, and

$$J_-^{ss}=\frac{k_{-1}k_{-2}k_{-3}}{k_1k_2+k_2k_3+k_3k_1+k_{-1}k_{-3}+k_{-2}k_{-3}+k_{-1}k_{-2}+k_1k_{-2}+k_2k_{-3}+k_3k_{-1}},$$

is the backward cycle flux.

The net cycle flux is just the Michaelis-Menten steady-state flux of (1), i.e.

$$v=\frac{k_Sc_S-k_Pc_P}{1+\frac{c_S}{K_{mS}}+\frac{c_P}{K_{mP}}},$$

where $k_S=\frac{k_1^0k_2k_3}{k_{-1}k_{-2}+k_{-1}k_3+k_2k_3}$, $k_P=\frac{k_{-1}k_{-2}k_{-3}^0}{k_{-1}k_{-2}+k_{-1}k_3+k_2k_3}$, $K_{mS}=\frac{k_{-1}k_{-2}+k_{-1}k_3+k_2k_3}{k_1^0(k_{-2}+k_2+k_3)}$, and $K_{mP}=\frac{k_{-1}k_{-2}+k_{-1}k_3+k_2k_3}{(k_{-2}+k_2+k_{-1})k_{-3}^0}$. That is just Eq. (2.46) in [4].

In addition, J_+^{ss} and J_-^{ss} can be rigorously proved to be the averaged numbers of the forward and backward cycles per time respectively due to ergodic theory [26, Theorem 2.1.2], i.e.

$$
\begin{aligned}
J^{ss} &= \lim_{t\to\infty} \frac{1}{t}\nu(t), \\
J_{+}^{ss} &= \lim_{t\to\infty} \frac{1}{t}\nu_{+}(t), \\
J_{-}^{ss} &= \lim_{t\to\infty} \frac{1}{t}\nu_{-}(t),
\end{aligned} \tag{7}
$$

where $\nu_+(t)$ and $\nu_-(t)$ are the number of occurrences of forward and backward cycles up to time t, and $\nu(t) = \nu_+(t) - \nu_-(t)$.

At the end of this section, it is important to notice that the quantity γ can be approximated by $\frac{\nu_+(t)}{\nu_-(t)}$ in single-molecule experiment when the time t is large enough, due to the fact that $\gamma = \frac{J_+^{ss}}{J_-^{ss}}$ and $J_+^{ss} = J_-^{ss}$ (i.e. $\gamma = 1$) if and only if this system is at chemical equilibrium.

3 Waiting cycle times and generalized Haldane equality

3.1 Mean waiting cycle times

The most obvious feature of the turnover trajectory (Fig. 1B in [29]) is its stochastic nature, exhibited both in the time needed for a chemical reaction which takes place on the subpicosecond time scale and that needed for diffusion and thermal activation which is much more longer. In the single-molecule experiment [29], the emission on-time and off-time recorded correspond to the "waiting time" for the turnover reactions, respectively. Once holding the statistical data of the trajectory in hand, the most straightforward analysis of the trajectories is certainly the distribution of the on-and-off times, so in our theoretical model, waiting cycle times should be defined and their mean should also be calculated at the first step.

Starting from the free enzyme state E, three kinds of waiting cycle times can be defined. T represents the waiting time for the occurrence of a forward or a backward cycle, T_+ represents the waiting time for the occurrence of a forward cycle, and T_- represents the waiting time for the occurrence of a backward cycle respectively. Obviously, T is just the smaller one of T_+ and T_-.

The problem of computing the mean waiting time $\langle T\rangle$ can be transferred into an important application of first-passage-time(FPT) methods (Fig.2) to the cyclic chemical transformations, in particular single-enzyme kinetics (Fig.1(b)).

FPT problems are been well studied, and there are analytical results for first-passage times in a discrete-time one-dimensional asymmetric random walk for quenched disorder [35]. But actually what we investigated in the present paper is the continuous-time case

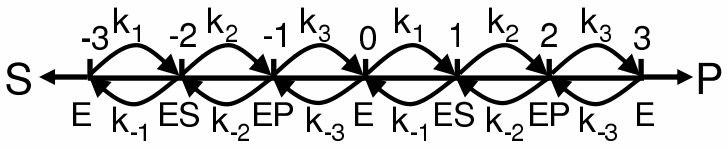

Figure 2: The kinetic scheme for computing the waiting cycle times. In order to distinguish the forward and backward cycles, Fig. 1 is transferred into a one-dimensional random walk model.

rather than the discrete-time case in [35], and the master equation (8) below is different from [35, Eq.(2.6)]. Therefore, the expression of $\langle T\rangle$ cannot be regarded as a particular case of the more general FPT problem when $M = L = 2$ in [35, Eq.(2.11)] although they are quite similar.

Let τ_i be the mean time first hitting the state 3 or -3 in Fig.2, starting from the state i. Obviously, $\langle T\rangle = \tau_0$ and $\tau_3 = \tau_{-3} = 0$.

Applying the strong Markov property of continuous-time Markov chains [1], $\{\tau_i\}$ satisfies the following equations

$$\begin{aligned}
\tau_{-2} &= \frac{1}{k_{-1}+k_2} + \frac{k_{-1}}{k_{-1}+k_2}\times 0 + \frac{k_2}{k_{-1}+k_2}\tau_{-1},\\
\tau_{-1} &= \frac{1}{k_{-2}+k_3} + \frac{k_{-2}}{k_{-2}+k_3}\tau_{-2} + \frac{k_3}{k_{-2}+k_3}\tau_0,\\
\tau_0 &= \frac{1}{k_{-3}+k_1} + \frac{k_{-3}}{k_{-3}+k_1}\tau_{-1} + \frac{k_1}{k_{-3}+k_1}\tau_1,\\
\tau_1 &= \frac{1}{k_{-1}+k_2} + \frac{k_{-1}}{k_{-1}+k_2}\tau_0 + \frac{k_2}{k_{-1}+k_2}\tau_2,\\
\tau_2 &= \frac{1}{k_{-2}+k_3} + \frac{k_{-2}}{k_{-2}+k_3}\tau_1 + \frac{k_3}{k_{-2}+k_3}\times 0.
\end{aligned} \tag{8}$$

Through simple calculation, one can get that

$$\langle T\rangle = \frac{k_1k_2+k_2k_3+k_3k_1+k_{-1}k_{-3}+k_{-2}k_{-3}+k_{-1}k_{-2}+k_1k_{-2}+k_2k_{-3}+k_3k_{-1}}{k_1k_2k_3+k_{-1}k_{-2}k_{-3}} = \frac{1}{J_+^{ss}+J_-^{ss}}.$$

Similarly, another mean waiting cycle time $\langle T_+\rangle$, which is the mean time to complete the forward cycle in Fig.1(b) whether before or after reaching an "analogous" final state in the opposite direction, can also be obtained as solutions of the nearly identical equations to (8) but with modified boundary conditions. Let τ_{i+} be the mean time first hitting the state 3, whether before or after the time hitting the state -3 in Fig.2, starting from the state i. Obviously, $\langle T_+\rangle = \tau_{0+}$, $\tau_{3+} = 0$ and $\tau_{-3+} = \tau_{0+}$.

Applying the strong Markov property of Markov chains again, $\{\tau_{i+}\}$ satisfies the following equations

$$\begin{aligned}
\tau_{-2+} &= \frac{1}{k_{-1}+k_2} + \frac{k_{-1}}{k_{-1}+k_2}\tau_{-3+} + \frac{k_2}{k_{-1}+k_2}\tau_{-1+},\\
\tau_{-1+} &= \frac{1}{k_{-2}+k_3} + \frac{k_{-2}}{k_{-2}+k_3}\tau_{-2+} + \frac{k_3}{k_{-2}+k_3}\tau_{0+},\\
\tau_{0+} &= \frac{1}{k_{-3}+k_1} + \frac{k_{-3}}{k_{-3}+k_1}\tau_{-1+} + \frac{k_1}{k_{-3}+k_1}\tau_{1+},\\
\tau_{1+} &= \frac{1}{k_{-1}+k_2} + \frac{k_{-1}}{k_{-1}+k_2}\tau_{0+} + \frac{k_2}{k_{-1}+k_2}\tau_{2+},\\
\tau_{2+} &= \frac{1}{k_{-2}+k_3} + \frac{k_{-2}}{k_{-2}+k_3}\tau_{1+} + \frac{k_3}{k_{-2}+k_3}\times 0,
\end{aligned} \tag{9}$$

which gives that

$$\langle T_+\rangle = \frac{k_1k_2+k_2k_3+k_3k_1+k_{-1}k_{-3}+k_{-2}k_{-3}+k_{-1}k_{-2}+k_1k_{-2}+k_2k_{-3}+k_3k_{-1}}{k_1k_2k_3} = \frac{1}{J_+^{ss}}.$$

Almost the same derivations can be achieved for $\langle T_-\rangle$, which is the mean time to complete the backward cycle in Fig.1(b), whether before or after reaching an "analogous" final state in the opposite direction, immediately follows

$$\langle T_-\rangle = \frac{k_1k_2+k_2k_3+k_3k_1+k_{-1}k_{-3}+k_{-2}k_{-3}+k_{-1}k_{-2}+k_1k_{-2}+k_2k_{-3}+k_3k_{-1}}{k_{-1}k_{-2}k_{-3}} = \frac{1}{J_-^{ss}}.$$

Surely, the expression of $\langle T_-\rangle$ can be directly derived due to the symmetry of the random walk in Fig. 2.

The quantitative relationship between the mean waiting cycle times ($\langle T\rangle$, $\langle T_+\rangle$, and $\langle T_-\rangle$) and the cycle fluxes (J^{ss}, J_+^{ss}, and J_-^{ss}) in this subsection is the first chief result of the present paper.

Consequently, $\langle T_+\rangle = \langle T_-\rangle$ if and only if this system is at chemical equilibrium, because of $\gamma = \frac{\langle T_-\rangle}{\langle T_+\rangle}$. Therefore, γ can also be measured by the ratio of averaged forward and backward waiting cycle times up to time t in the single-molecule experiment, which is different from the measure method introduced at the end of the previous subsection. Nonetheless, applying the elementary renewal theorem [8, Sec.3.4, Theorem 4.1,4.2], the two methods are asymptotically the same because $\langle T_+\rangle \approx \frac{t}{\nu_+(t)}$ and $\langle T_-\rangle \approx \frac{t}{\nu_-(t)}$ when t is large.

3.2 Stepping probability

The stepping probabilities $p^+(t)$ and $p^-(t)$ up to time t are just the fractions of $\nu_+(t)$ and $\nu_-(t)$, representing the weights of the forward and backward cycles respectively from the statistical point of view in experiments, i.e.

$$p^+(t) = \frac{\nu_+(t)}{\nu_+(t)+\nu_-(t)}, \quad p^-(t) = \frac{\nu_-(t)}{\nu_+(t)+\nu_-(t)}.$$

According to Eq.7, one can get the eventual stepping probability

$$\begin{aligned} p^+ &\stackrel{def}{=} \lim_{t\to\infty} p^+(t) = \frac{J_+^{ss}}{J_+^{ss}+J_-^{ss}} = \frac{k_1k_2k_3}{k_1k_2k_3+k_{-1}k_{-2}k_{-3}}, \\ p^- &\stackrel{def}{=} \lim_{t\to\infty} p^-(t) = \frac{J_-^{ss}}{J_+^{ss}+J_-^{ss}} = \frac{k_{-1}k_{-2}k_{-3}}{k_1k_2k_3+k_{-1}k_{-2}k_{-3}}. \end{aligned} \tag{10}$$

It is necessary to point out that the stepping probabilities $p^+(t)$ and $p^-(t)$ are random variables depending on the trajectories, while their fluctuations tend to vanish when t tends to infinity. Hence the eventual stepping probability p^+ and p^- are independent with the trajectories due to the ergodic theory.

Interesting, the forward stepping probability can also be defined as $p^+ \stackrel{def}{=} P_{\{E\}}(T_+ < T_-)$, which means the probability that the particle first completes a forward cycle before a backward one starting from the initial free enzyme E. Similarly, the backward stepping probability can be defined as $p^- \stackrel{def}{=} P_{\{E\}}(T_- < T_+)$. This is the second chief result of the present article.

This equivalence can be explicitly seen through translating this problem to a corresponding one of the random walk in Fig. 2, either.

Let p_{i+} be the probability of hitting the state 3 before -3 in Fig.2, starting from the state i. Obviously, $p_{3+} = 1$ and $p_{-3+} = 0$.

Again applying the strong Markov property of Markov chains as what we have done in the precious section, $\{p_{i+}\}$ satisfies the following equations

$$\begin{aligned} p_{-2+} &= \frac{k_{-1}}{k_{-1}+k_2}\times 0 + \frac{k_2}{k_{-1}+k_2}p_{-1+}, \\ p_{-1+} &= \frac{k_{-2}}{k_{-2}+k_3}p_{-2+} + \frac{k_3}{k_{-2}+k_3}p_{0+}, \\ p_{0+} &= \frac{k_{-3}}{k_{-3}+k_1}p_{-1+} + \frac{k_1}{k_{-3}+k_1}p_{1+}, \\ p_{1+} &= \frac{k_{-1}}{k_{-1}+k_2}p_{0+} + \frac{k_2}{k_{-1}+k_2}p_{2+}, \\ p_{2+} &= \frac{k_{-2}}{k_{-2}+k_3}p_{1+} + \frac{k_3}{k_{-2}+k_3}\times 1. \end{aligned}$$

Through simple calculation, one can get that

$$p^+ = P_{\{E\}}(T_+ < T_-) = p_{0+} = \frac{k_1k_2k_3}{k_1k_2k_3 + k_{-1}k_{-2}k_{-3}},$$

and

$$p^- = P_{\{E\}}(T_+ > T_-) = 1 - P_{\{E\}}(T_+ < T_-) = \frac{k_{-1}k_{-2}k_{-3}}{k_1k_2k_3 + k_{-1}k_{-2}k_{-3}}.$$

Consequently,

$$p^+ = \frac{J_+^{ss}}{J_+^{ss} + J_-^{ss}} = \frac{\langle T\rangle}{\langle T_+\rangle},$$

$$p^- = \frac{J_-^{ss}}{J_+^{ss} + J_-^{ss}} = \frac{\langle T\rangle}{\langle T_-\rangle},$$

and

$$\triangle\mu = k_BT\log\gamma = k_BT\log\frac{p^+}{p^-} = k_BT\log\frac{J_+^{ss}}{J_-^{ss}} = k_BT\log\frac{\langle T_-\rangle}{\langle T_+\rangle},$$

which follows $p^+ = p^-$ if and only if this system is at chemical equilibrium.

3.3 Generalized Haldane equality

To avoid the unnecessary difficult mathematical details, we apply a simple trick like the "time-reversal mapping" always used in modern statistical physics [5, 6, 7, 22, 23, 24, 25] instead of the rigorous language of measure theory.

We introduce a one-to-one mapping r for the trajectory of the simple kinetic in Fig. 1, which belongs to the event $\{T_+ < T_-\}$, mapped to its "quasi-time-reversal" one.

For each trajectory $\omega = \{\omega_t : t \geq 0,\ \omega_0 = \{E\}\}$ belonging to the set $\{T_+ < T_-\}$, let T^* be the *last time* when it leaves the state $\{E\}$ before finishing a forward cycle in the Fig.1(b). Then its "quasi-time-reversal" one $r\omega = \{(r\omega)_t : t \geq 0\}$ is defined as follows:
i) when the time t is before or equal to T^*, then one just copy ω to $r\omega$, i.e.$(r\omega)_t = \omega_t$;
ii) when the time t is between T^* and T_+, then one maps the real time-reversal trajectory of ω with respect to the time interval $[T^*, T_+]$ to $r\omega$, i.e. $(r\omega)_t = \omega_{T^*+T_+-t}$;
iii) when the time t is greater than T_+, then one can also simply copy ω to $r\omega$ as what we have done in (i).

See Fig. 3 for an illustrative example. As having been pointed out on this figure, T^* is denoted to be the *last time* when it leaves the state $\{E\}$ before finishing a forward cycle $E \to ES \to EP \to E$. Then *the ratio* of the probability density of the above trajectory with respect to its "quasi-time-reversal" one below is

$$\gamma = \frac{(k_1k_{-1}k_{-3}k_3) \times (k_1k_2k_{-2}k_2k_3)}{(k_1k_{-1}k_{-3}k_3) \times (k_{-3}k_{-2}k_2k_{-2}k_{-1})} = \frac{k_1k_2k_3}{k_{-1}k_{-2}k_{-3}}.$$

Now it is indispensable to explain why we construct the above mapping like this.

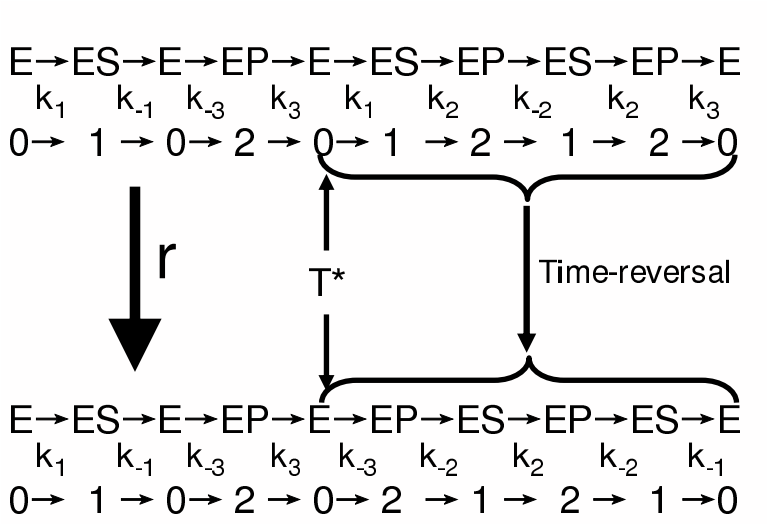

Figure 3: An illustrative example of the "quasi-time-reversal" map. T^* is the *last time* when it leaves the state $\{E\}$ before finishing a forward cycle $E \to ES \to EP \to E$, then one maps the real time-reversal trajectory of ω with respect to the time interval $[T^*, T_+]$ to $r\omega$. See text for details.

1) The number of the steps $E \to ES$ in the original trajectory ω belonging to $\{T_+ < T_-\}$ is one more than that in its "quasi-time-reversal" corresponding trajectory $r\omega$ belonging to $\{T_+ > T_-\}$, while the number of the steps $ES \to E$ in ω is one less than that in $r\omega$;

similarly,

2) The number of the steps $ES \to EP$ in the trajectory ω is one more than that in $r\omega$, while the number of the steps $EP \to ES$ in the trajectory ω is one less than that in $r\omega$;

3) The number of the steps $EP \to E$ in the trajectory ω is one more than that in $r\omega$, while the number of the steps $E \to EP$ in the trajectory ω is one less than that in $r\omega$;

and more important

4) The dwell time upon each state of the trajectory ω and its "quasi-time-reversal" corresponding one $r\omega$ is mapped quite well such that the difference between ω and $r\omega$ are only exhibited upon their sequences of states.

Consequently, the most important observation is that *the ratio* of the probability density of each trajectory ω in $\{T_+ < T_-\}$ with respect to its "quasi-time-reversal" trajectory $r\omega$ in $\{T_+ > T_-\}$ is invariable, which is surprisingly always equal to the constant $\gamma = \frac{k_1 k_2 k_3}{k_{-1} k_{-2} k_{-3}}$.

Rigorous proof needs to be expressed in the language of measure theory, especially

applying the Radon-Nikodym derivative similar to [26, Lemma 2.2.7], so more details is omitted here.

Furthermore, the map r is a one-to-one correspondence between the trajectory sets $\{T_+ < T_-\}$ and $\{T_+ > T_-\}$. More particularly, for each $t \geq 0$, the map r is also actually a one-to-one correspondence between the trajectory sets $\{T_+ = t < T_-\}$ and $\{T_+ > T_- = t\}$.

Therefore, for each $t \geq 0$,

$$P_{\{E\}}(T_+ = t, T_+ < T_-) = \gamma P_{\{E\}}(T_- = t, T_- < T_+),$$

and

$$p^+ = P_{\{E\}}(T_+ < T_-) = \gamma P_{\{E\}}(T_- < T_+) = \gamma p^-,$$

which has already been proved in the above section.

Denote the conditional probability density of T_+ given that $\{T_+ < T_-\}$ as $\Theta_+(t) = P_{\{E\}}(T_+ = t | T_+ < T_-)$, and the conditional probability density of T_- given that $\{T_- < T_+\}$ as $\Theta_-(t) = P_{\{E\}}(T_- = t | T_- < T_+)$. Hence,

$$\begin{aligned} \Theta_+(t) &= P_{\{E\}}(T_+ = t | T_+ < T_-) = \frac{P_{\{E\}}(T_+ = t, T_+ < T_-)}{P_{\{E\}}(T_+ < T_-)} \\ &= \frac{\gamma P_{\{E\}}(T_- = t, T_- < T_+)}{\gamma P_{\{E\}}(T_- < T_+)} = P_{\{E\}}(T_- = t | T_- < T_+) = \Theta_-(t), \ \forall t. \end{aligned} \quad (11)$$

And also denote the probability density of T as $\Theta(t) = P_{\{E\}}(T = t)$, so

$$\Theta(t) = \Theta_+(t)p^+ + \Theta_-(t)p^- = \Theta_+(t) = \Theta_-(t).$$

It consequently follows a very important corollary that the distribution of waiting cycle time T is *independent* of whether the enzyme E completes a forward cycle or a backward cycle, although the probability of these two cycles might be rather different, i.e.

$$P_{\{E\}}(T = t, T_+ < T_-) = P(T_+ = t, T_+ < T_-) = \Theta_+(t)p^+ = \Theta(t)p^+,$$

and

$$P_{\{E\}}(T = t, T_+ > T_-) = P(T_- = t, T_+ < T_-) = \Theta_-(t)p^- = \Theta(t)p^-.$$

Furthermore, we have

$$\langle T_+, T_+ < T_- \rangle = p^+ \langle T \rangle,$$

$$\langle T_-, T_- < T_+ \rangle = p^- \langle T \rangle,$$

and

$$\langle T_+ | T_+ < T_- \rangle = \langle T_- | T_- < T_+ \rangle = \langle T \rangle,$$

which means even in the far from equilibrium case ($\gamma >> 1$), the dwell times for each forward cycle or each backward cycle are identical although their frequencies may be rather different ($p^+ >> p^-$).

At the end of this section, we will present an interesting corollary about the entropy production rate e_p. Due to the classical result of entropy production rate in a general mesoscopic model of biochemical kinetic diagrams [19, 20, 26], one has

$$e_p = (J_+^{ss} - J_-^{ss}) \log \gamma,$$

where $\log \gamma = \log \frac{J_+^{ss}}{J_-^{ss}}$ is the entropy production rate of the cycle $E \to ES \to EP \to E$, and $J_+^{ss} - J_-^{ss}$ is its net cycle flux.

Applying the above results to waiting cycle times, e_p can be expressed as

$$\begin{aligned} e_p &= (\frac{1}{\langle T_+ \rangle} - \frac{1}{\langle T_- \rangle}) \log \gamma \\ &= (\frac{p^+}{\langle T \rangle} - \frac{p^-}{\langle T \rangle}) \log \gamma \\ &= (p^+ - p^-) avepr, \end{aligned} \tag{12}$$

where $avepr = \frac{1}{\langle T \rangle} \log \gamma = \frac{1}{\langle T \rangle} \log \frac{J_+^{ss}}{J_-^{ss}} = \frac{1}{\langle T \rangle} \log \frac{\langle T_- \rangle}{\langle T_+ \rangle} = \frac{1}{\langle T \rangle} \log \frac{p^+}{p^-}$ is regarded as the time-averaged entropy production rate of the cycle $E \to ES \to EP \to E$.

Finally, it should be emphasized that this entropy production rate can also be measured by $(\nu_+(t) - \nu_-(t)) \log \frac{\nu_+(t)}{\nu_-(t)}$ when the time t is large in the single-molecule experiment, recalling that J_+^{ss} and J_-^{ss} can be approximated by $\nu_+(t)$ and $\nu_-(t)$ respectively.

4 Extending to the n-step cycle

In the previous section, most of the results are obtained through solving a number of master equations similar to (8). But now we claim that the same results can be extended to the n-step cycle [14], according the elementary renewal theorem in probability theory [8, Sec. 3.4, Theorem 4.1,4.2] and general circulation theory of Markov chains [26, Chapter 1,2], which has already been derived for more than two decades. But the key method is also the same "quasi-time-reversal" mapping r introduced in the previous section.

Below is the summation of the main results in the n-step cycle, which is quite similar to the 3-step cycle.

4.1 Cycle flux and NESS

We consider a n-step mechanism of the Michaelis-Menten kinetics in which the conversion of S into P in the catalytic site of the enzyme is represented as a process separate from

release of P from the enzyme.

$$E+S \underset{k_{-1}}{\overset{k_1^0}{\rightleftharpoons}} ES(=ES_1) \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ES_2 \cdots \underset{k_{-(n-1)}}{\overset{k_{n-1}}{\rightleftharpoons}} EP(=ES_{n-1}) \underset{k_{-n}^0}{\overset{k_n}{\rightleftharpoons}} E+P, \tag{13}$$

in which k_1^0 and k_{-3}^0 are second-order rate constants.

If there is only one enzyme molecule, then from the enzyme perspective, the kinetics are stochastic and cyclic, with the pseudo-first-order rate constants $k_1 = k_1^0 c_S$ and $k_{-n} = k_{-n}^0 c_P$ where c_S and c_P are the sustained concentrations of substrate S and P in the steady state.

This system is at chemical equilibrium if and only if

$$\frac{k_1 k_2 k_3 \cdots k_n}{k_{-1} k_{-2} k_{-3} \cdots k_{-n}} = 1. \tag{14}$$

In the nonequilibrium case,

$$\frac{k_1 k_2 k_3 \cdots k_n}{k_{-1} k_{-2} k_{-3} \cdots k_{-n}} = \gamma \neq 1, \tag{15}$$

and $\triangle\mu = k_B T \ln \gamma$ is well known as the cellular phosphorylation potential.

Denote a n-dimensional matrix $Q = \{q_{ij}\}_{n\times n}$ in which $q_{i,i+1} = k_i$, $q_{i,i-1} = k_{-(i-1)}$, $i = 2, \cdots, n-1$, $q_{1,2} = k_1, q_{n,1} = k_n, q_{1,n} = k_{-n}, q_{n,n-1} = k_{-(n-1)}$, and others are all zero. And let $D(H)$ be the determinant of Q with rows and columns indexed by the index set H.

Then according to [26, Theorem 2.1.2], the enzyme turnover rate of $S \to P$, which corresponds to the net flux of the n-step cycle, can be expressed as

$$J^{ss} = \frac{k_1 k_2 k_3 \cdots k_n - k_{-1} k_{-2} k_{-3} \cdots k_{-n}}{\sum_{i=1,2,\cdots,n} D(\{i\}^c)} = J_+^{ss} - J_-^{ss}, \tag{16}$$

where

$$J_+^{ss} = \frac{k_1 k_2 k_3 \cdots k_n}{\sum_{i=1,2,\cdots,n} D(\{i\}^c)},$$

is the forward cycle flux, and

$$J_-^{ss} = \frac{k_{-1} k_{-2} k_{-3} \cdots k_{-n}}{\sum_{i=1,2,\cdots,n} D(\{i\}^c)},$$

is the backward cycle flux.

It should be noticed that the expression of $\sum_{i=1,2,\cdots,n} D(\{i\}^c)$ is equivalent to the King-Altman method [4, Chapter 4] but more general and applicable. Furthermore, it can be easy simulated by mathematical softwares, such as Matlab and Mathematica.

The net cycle flux can also be expressed as the Michaelis-Menten steady-state flux of (13), i.e.

$$v = \frac{k_S c_S - k_P c_P}{1 + \frac{c_S}{K_{mS}} + \frac{c_P}{K_{mP}}},$$

where the definitions of k_S, k_P, K_{mS}, and K_{mP} are much more complicated than the 3-step cycle. That is just Eq. (2.46) in [4].

Also similar to the 3-step cycle, J_+^{ss} and J_-^{ss} can be rigorously proved to be the averaged numbers of the forward and backward cycles per time respectively due to ergodic theory [26, Theorem 2.1.2], i.e.

$$\begin{aligned} J^{ss} &= \lim_{t\to\infty} \frac{1}{t}\nu(t), \\ J_+^{ss} &= \lim_{t\to\infty} \frac{1}{t}\nu_+(t), \\ J_-^{ss} &= \lim_{t\to\infty} \frac{1}{t}\nu_-(t), \end{aligned} \tag{17}$$

where $\nu_+(t)$ and $\nu_-(t)$ are the number of occurrences of forward and backward cycles up to time t, and $\nu(t) = \nu_+(t) - \nu_-(t)$.

4.2 Mean waiting cycle times

Starting from the free enzyme state E, three kinds of waiting cycle times can be defined. T represents the waiting time for the occurrence of a forward or a backward cycle, T_+ represents the waiting time for the occurrence of a forward cycle, and T_- represents the waiting time for the occurrence of a backward cycle respectively.

According to the elementary renewal theorem [8, Sec.3.4, Theorem 4.1,4.2],

$$\langle T\rangle = \lim_{t\to\infty} \frac{1}{\nu_+(t)+\nu_-(t)} = \frac{1}{J_+^{ss}+J_-^{ss}}.$$

Similarly,

$$\langle T_+\rangle = \lim_{t\to\infty} \frac{1}{\nu_+(t)} = \frac{1}{J_+^{ss}},$$

and

$$\langle T_-\rangle = \lim_{t\to\infty} \frac{1}{\nu_-(t)} = \frac{1}{J_-^{ss}}.$$

4.3

The stepping probabilities $p^+(t)$ and $p^-(t)$ up to time t are just the fractions of $\nu_+(t)$ and $\nu_-(t)$ from the statistical point of view in experiments, i.e.

$$p^+(t) = \frac{\nu_+(t)}{\nu_+(t)+\nu_-(t)}, \quad p^-(t) = \frac{\nu_-(t)}{\nu_+(t)+\nu_-(t)}.$$

According to Eq.17, one can get the eventual stepping probability

$$\begin{aligned} p^+ &\overset{def}{=} \lim_{t\to\infty} p^+(t) = \frac{J_+^{ss}}{J_+^{ss}+J_-^{ss}}, \\ p^- &\overset{def}{=} \lim_{t\to\infty} p^-(t) = \frac{J_-^{ss}}{J_+^{ss}+J_-^{ss}}. \end{aligned} \tag{18}$$

Moreover, due to Eq.(19) in the next subsection, one can prove that the forward stepping probability could also be defined as $p^+ \overset{def}{=} P_{\{E\}}(T_+ < T_-)$, which means the probability that the particle first completes a forward cycle before a backward one, starting from the initial state $\{E\}$. Similarly, the backward stepping probability could be defined as $p^- \overset{def}{=} P_{\{E\}}(T_- < T_+)$, too.

Consequently,

$$p^+ = \frac{J_+^{ss}}{J_+^{ss} + J_-^{ss}} = \frac{\langle T \rangle}{\langle T_+ \rangle},$$

$$p^- = \frac{J_-^{ss}}{J_+^{ss} + J_-^{ss}} = \frac{\langle T \rangle}{\langle T_- \rangle},$$

and

$$\triangle \mu = k_B T \log \frac{p^+}{p^-} = k_B T \log \frac{J_+^{ss}}{J_-^{ss}} = k_B T \log \frac{\langle T_- \rangle}{\langle T_+ \rangle}.$$

4.4 Generalized Haldane equality

Also introduce the same one-to-one mapping r for the trajectory of the n-step kinetic, which belongs to the event $\{T_+ < T_-\}$, mapped to its "quasi-time-reversal" one.

And recall that the number of each forward step in the original trajectory ω belonging to $\{T_+ < T_-\}$ of the n-step model is one more than that in its corresponding quasi-time-reversal trajectory $r\omega$ belonging to $\{T_+ > T_-\}$, and on the contrary the number of each backward steps in ω is one less than that in $r\omega$. And the dwell times of the trajectory ω and $r\omega$ are mapped quite well such that the difference between ω and $r\omega$ are only exhibited in their sequences of states.

Consequently, the most important observation is that the ratio of the probability density of every trajectory ω in $\{T_+ < T_-\}$ with respect to its "quasi-time-reversal" $r\omega$ in $\{T_+ > T_-\}$ is invariable, which is surprisingly always equal to the constant $\gamma = \frac{k_1 k_2 k_3 \cdots k_n}{k_{-1} k_{-2} k_{-3} \cdots k_{-n}}$.

On the other hand, the trajectory map r is a one-to-one correspondence between the trajectory sets $\{T_+ < T_-\}$ and $\{T_+ > T_-\}$. More particularly, for each $t \geq 0$, the map r is also actually a one-to-one correspondence between the trajectory sets $\{T_+ = t < T_-\}$ and $\{T_+ > T_- = t\}$.

Therefore,

$$p^+ = \gamma p^-, \tag{19}$$

which can be used to prove the results of stepping probabilities in the previous subsection. Then one arrives at the generalized Haldane equality in the version of distribution,

$$\Theta_+(t) \;\; = \;\; P_{\{E\}}(T_+ = t | T_+ < T_-) = P_{\{E\}}(T_- = t | T_- < T_+) = \Theta_-(t), \;\; \forall t \geq 0, \tag{20}$$

and

$$\Theta(t) = P_{\{E\}}(T = t) = \Theta_+(t)p^+ + \Theta_-(t)p^- = \Theta_+(t) = \Theta_-(t).$$

It consequently follows a very important corollary that the distribution of waiting cycle time T is *independent* of whether the enzyme E completes a forward cycle or a backward cycle, although the probability of these two cycles might be rather different.

Hence, the generalized Haldane equality in the version of conditional expectation is

$$\langle T_+ | T_+ < T_- \rangle = \langle T_- | T_- < T_+ \rangle = \langle T \rangle, \tag{21}$$

which means even in the far from equilibrium case, the dwell times for each forward cycle or each backward cycle are the same although their frequencies might be rather different.

Similar to the previous section, we present an interesting corollary about the entropy production rate $e_p = (J_+^{ss} - J_-^{ss}) \log \gamma$, and

$$\begin{aligned} e_p &= (\frac{1}{\langle T_+ \rangle} - \frac{1}{\langle T_- \rangle}) \log \gamma \\ &= (\frac{p^+}{\langle T \rangle} - \frac{p^-}{\langle T \rangle}) \log \gamma \\ &= (p^+ - p^-) avepr, \end{aligned} \tag{22}$$

where $avepr = \frac{1}{\langle T \rangle} \log \gamma = \frac{1}{\langle T \rangle} \log \frac{J_+^{ss}}{J_-^{ss}} = \frac{1}{\langle T \rangle} \log \frac{\langle T_- \rangle}{\langle T_+ \rangle} = \frac{1}{\langle T \rangle} \log \frac{p^+}{p^-}$ is regarded as the time-averaged entropy production rate of the n-step cycle.

Furthermore, the following statements are equivalent to each other:

1) This n-step system is at chemical equilibrium;

2) The cellular phosphorylation potential $\triangle\mu$ vanishes, i.e. $\gamma = 1$;

3) The forward and backward cycle fluxes are identical, i.e. $J_+^{ss} = J_-^{ss}$;

4) The waiting times for the forward and backward cycles are identical, i.e. $\langle T_+ \rangle = \langle T_- \rangle$;

5) The eventual stepping probabilities of the forward and backward cycles $p^+ = p^-$.

Finally, it should be emphasized that this entropy production rate e_p can also be measured by $(\nu_+(t) - \nu_-(t)) \log \frac{\nu_+(t)}{\nu_-(t)}$ when the time t is large in the single-molecule experiment, recalling that J_+^{ss} and J_-^{ss} can be approximated by $\nu_+(t)$ and $\nu_-(t)$ respectively.

5 Experimental and theoretically based evidence

Several results proved in the present article have been observed and discovered in the recently reported single-molecule experiment [3] of kinesin, which is one of the most important molecular motor proteins. The time trajectories of single kinesin molecules have been measured for different external forces and for different ATP concentrations, recording the number of forward and backward steps $(\nu_+(t),\ \nu_-(t))$ and the stepping probabilities

$(p^+(t),\ p^-(t))$, which are also called fractions of forward and backward steps. They investigated the physical mechanism of the kinesin step, and found that both forward and backward 8-nm steps occur on the microsecond timescale without mechanical substeps on this timescale. It was also shown in [3, Fig. 3] that the time constants for the ensemble averaged forward and backward steps are very similar, which is just the generalized Haldane equality) in the version of conditional expectation (21). However, this interesting observation has not attached enough importance to in [3] and there was no theoretical analysis either.

Almost published at the same time, Kolomeisky, A.B., et.al. [27] put forward a discrete-time biased random walk model, applying the first-passage-time method [35] and splitting probability theory [47, Chap. XI], to provide explicit expressions for the fractions of forward and backward steps and dissociations, and most important to conclude that the mean dwell times to move forward, backward, or irreversible detach are equal to each other, independent of ATP concentrations or external forces [27, Fig.3]. The concept of splitting probability in [27, 47] is the same as the stepping probability in the present paper, and the mean dwell times to move forward and backward in the discrete-time model in [27] just correspond to the conditional expectation of T_+ given $T_+ < T_-$ and the conditional expectation of T_- given $T_- < T_+$ in the continuous-time model of the present paper respectively, hence what they conclude was also the generalized Haldane equality in the version of conditional expectation (Eq. 21). They also claimed that these forward and backward dwell times should be independent of what direction the motor protein will go in the next step, although the probability of these steps might be rather different. Nonetheless, they didn't notice the forward and backward fluxes and generalized Haldane equality in the version of distribution (20), and it is a pity that they didn't consider the continuous-time case which is actually more difficult to prove.

Meanwhile, Kou, S.C., et.al. [28] summarized their theoretical understanding of single-molecule kinetics, and focused on the conditions under which a single-molecule Michaelis-Menten equation for the reciprocal of the mean stochastic waiting time T for individual turnovers ([28, Eqs. 14, 26, 30, 34]). As have been mentioned in the introduction, the model they built is the simplest irreversible Michaelis-Menten mechanism, and the state space of their stochastic model (Markov chain) actually only contains two states (E and ES), which is just why they can just only use the ordinary differential equations to get the explicit distribution function $f(t)$ of the waiting time or its approximations, avoiding to apply the strong Markov property which is the basic method to compute the first-passage-time problems in stochastic processes. So their method can not be generalized to more complicated cases, and generally speaking, the explicit distribution function $f(t)$ can

rarely be analytically obtained in a multi-state and reversible stochastic model. Moreover, they didn't notice the existence of forward and backward waiting cycle times T_+ and T_-, and neglected many insightful observations.

Afterwards, Qian, H. and Xie, X.S.[41] studied a semi-Markov model of single-enzyme turnover in nonequilibrium steady states with sustained concentrations of substrates and products, since in some sense, the general result for *any* enzyme kinetics, if there is only one free enzyme state, can be mapped to a semi-Markov process. Then they gave a brief proof to the generalized Haldane equality in the version of distribution (20) and also expressed the nonzero chemical driving force $\triangle\mu$ as $k_BT\log\frac{\nu_+(t)}{\nu_-(t)}$. Hence a part of our results in the present paper are a special case of the rigorous semi-Markov result that Wang and Qian have obtained by the elementary renewal theorem [40, 48]. But they didn't explicitly distinguish the *conditional* waiting cycle time and the *absolute* waiting cycle times, and may cause some ambiguities.

At the end of this section, what should be paid more attention to in the statistical data analysis of the experiment is to distinguish the data of waiting cycle times T, T_+ and T_-. In reference [3], they only recorded the data of T, and divided them into two classes according to whether have completed a forward or a backward cycle. Consequently they found the mean values of the data in these two classes are very similar. But they haven't realized that the distributions of the data in these two classes should also be very similar, and actually they are data of the conditional waiting cycle times rather than the absolute waiting cycle times T, T_+ and T_-, because in the nonequilibrium case, $\langle T_+\rangle$ and $\langle T_-\rangle$ can not be identical and must be both greater than $\langle T\rangle$. See Fig. 4 for an illustrative example.

According to the strong Markov property in the theory of stochastic processes, we claim that the data of T_+ and T_- can also be obtained from the same trajectory recorded in the experiment (Fig. 4), and we believe that other main results in the present paper can also be discovered from the same experiment data, especially the relationships between the cycle fluxes, mean cycle times and eventual stepping probabilities, which we have summarized in the previous section.

6 Discussion

Deterministic, nonlinear mathematical models usually based on the law of mass action have been traditionally used for modelling biological systems [10, 33], while nowadays stochastic fluctuations observed in most living organisms, such as evidences in the single-molecule approach [29, 2], have changed the way biophysical or biochemical problems are presented and have been recognized as a major important effect in cell biology. Stochastic models in

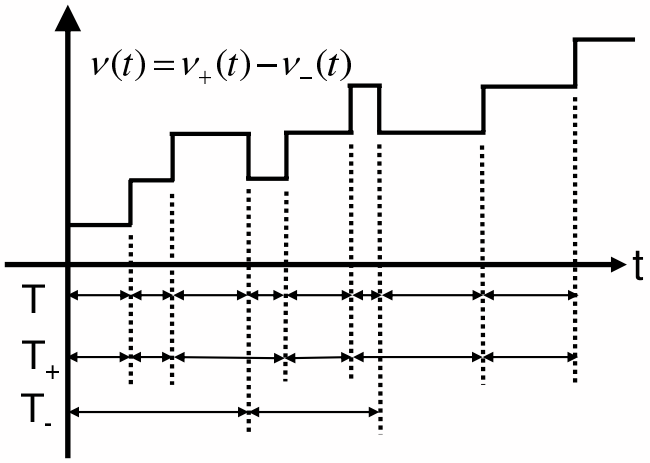

Figure 4: The solid line illustrates ideal data on single-enzyme cycling as a function of time, with distinguished data analysis of T, T_+ and T_-. See text for details.

biochemistry have already provided important insights and quantitative characterizations of a wide range of biochemical systems [19, 30, 47, 11, 12, 39, 52].

In single-molecule experiments, the microscopic motion of an enzyme molecule undergoes rapid thermal fluctuation due to its incessant collisions with the solvent molecules, and therefore, the data obtained are inevitably stochastic [49]. The main results in the present paper are actually based on one type of measurements in single-molecule enzymology, which records the stochastic conformational dynamics of an enzyme turnover (called "*trajectories*") [29]. From the perspective of the stochastic process, a trajectory is a stationary stochastic process which can be analyzed by statistical methods. And moreover based on the ergidic theory, which is an elementary law in both statistical physics and the mathematical theory of stochastic process, an arbitrary single trajectory surprisingly contains all the information of the stochastic system.

However, it is often thought that the noise added to the biological models only provides moderate refinements to the behaviors otherwise predicted by the classical deterministic system description, while in the present paper, it is quite obvious that the main problems discussed here are *impossible* even to be put forward in a deterministic model. So it may be necessary to reconstruct the main biological theory based on the stochastic models in order to explain the experiment results of single molecule tracking.

For instance, applying the statistical methods to analysis the recorded single trajectory, experimentalists can not only directly measure the distribution of the waiting cycle (turnover) times T, T_+ and T_-, but also the probability cycle fluxes J^{ss}, J_+^{ss} and J_-^{ss}

widely used in this paperwhich can be approximated by the time-averaged number of occurrences of the cycle, according to the general ergodic theory of cycle fluxes [26, Theorem 2.1.2] and elementary renewal theorem [8, Sec. 3.4, Theorem 4.1,4.2]. Furthermore, it is important to notice that the distributions of single-molecule properties and the probability cycle fluxes can only be presented and measured through single-molecule experiments rather than the ensemble average.

In conclusion, the single-molecule enzymology is still in its early ages, and in my personal opinion, the generalized Haldane equality as well as the relationship between mean waiting cycle times and cycle fluxes may be the first interesting discovery in this active field, which could be applied to instruct the data analysis of single-molecule trajectories. In the future, we believe that more and more important phenomenon and theory in traditional enzymology, such as inhibition and activation, cooperativity and multi-enzyme systems, will enter into the single-molecule enzymology, which might stimulate very significant developments both in experiment and theory.

In addition, from the purely mathematical point of view, we also believe that it is valuable to further extend similar results to the theory of much more general Markov chains, though much technical work remains to be done.

Acknowledgement

The authors would like to thank Prof. Hong Qian at University of Washington for introducing this problem, and Prof. Min Qian, Prof. Maozheng Guo, Prof. Daquan Jiang and Yuping Zhang in Peking University for helpful discussion in our seminar. This work is partly supported by the NSFC (Nos. 10701004, 10531070 and 10625101) and 973 Program 2006CB805900.

References

[1] Anderson, W.J. : *Continuous-time Markov chains: an applications-oriented approach.* New York: Springer-Verlag 1991

[2] Bai, C.L., Wang, C., Xie, X.S. and Wolynes, P.G.: Single molecule physics and chemistry. *Proc. Natl. Acad. Sci. USA* **96** 11075-11076 (1999)

[3] Carter, N.J. and Cross, R.A.: Mechanics of the kinesin step. *Nature.* **435** 308-312 (2005)

[4] Cornish-Bowden, A.: *Fundamentals of enzyme kinetics.* (3nd ed.) London: Portland Press 2004

[5] Crooks, G.E.: Nonequilibrium measurements of free energy differences for microscopically reversible markovian systems. *J. Stat. Phys.* **90**, 1481–1487 (1998)

[6] Crooks, G.E.: Entropy production fluctuation theorem and the nonequilibrium work relation for free energy differences. *Phys. Rev. E* **60**, 2721–2726 (1999)

[7] Crooks, G.E.: Path-ensemble averages in systems driven far from equilibrium. *Phys. Rev. E* **61**(3), 2361-2366 (2000)

[8] Durrett, R.: *Probability: Theory and Examples* (Second Edition). Pacific Grove, CA: Duxbury Press 1996

[9] English, B.P., Min, W., van Oijen, A.M., Lee, K.T.; Luo, G., Sun, H., Cherayil, B.J., Kou, S.C. and Xie, X.S.: Ever-fluctuating single enzyme molecules: Michaelis-Menten equation revisited. *Nat. Chem. Bio.* **2** 87-94 (2006)

[10] Fall, C.P., Marland, E.S., Wagner, J.M. and Tyson, J.J.: *Computational cell biology.* New York: Springer-Verlag 2002

[11] Fox, R.F. and Lu, Y.: Emergent collective behavior in large numbers of globally coupked independently stochastic ion channels. *Phys. Rev. E* **49**(4) 3421-3431 (1994)

[12] Fox, R.F.: Stochastic versions of the Hodgkin-Huxley equations. *Biophys. J.* **72** 2068-2074 (1997)

[13] Ge, H., Qian, H. and Qian, M.: Synchronized dynamics and nonequilibrium steady states in a yeast cell-cycle network. *Math. Biosci.* To appear.

[14] Mathematical rigorous proof based on measure theory is being prepared.

[15] Glansdorff, P. and Prigogine, I.: *Thermodynamic theory of structure, stability and fluctuations.* London: Wiley-Interscience 1971

[16] Haken, H.: *Synergetics: an introduction: nonequilibrium phase transitions and self-organization in physics, chemistry, and biology.* Berlin, New York: Springer-Verlag 1977

[17] Haken, H.: *Advanced synergetics: instability hierarchies of self-organizing systems and devices.* Berlin, New York: Springer-Verlag 1983

[18] Hill, T.L.: Studies in irreversible thermodynamics IV. diagrammatic representation of steady state fluxes for unimolecular systems. *J. Theoret. Biol.* 10, 442-459 (1966)

[19] Hill, T.L.: *Free energy transduction in biology.* New York: Academic Press 1977

[20] Hill, T.L.: *Free energy transduction and biochemical cycle kinetics.* New York: Springer-Verlag 1995

[21] Hill, T. and Chen, Y.: Stochastics of cycle completions (fluxes) in biochemical kinetic diagrams. *Proc. Nat. Acad. Sci. USA* 72, 1291–1295(1975)

[22] Jarzynski, C.: Nonequilibrium equality for free energy differences. *Phys. Rev. Lett.* **78**, 2690–2693 (1997)

[23] Jarzynski, C.: Equilibrium free-energy differences from nonequilibrium measurements: A master-equation approach. *Phys. Rev. E* **56**, 5018–5035 (1997)

[24] Jarzynski, C.: Microscopic analysis of Clausius-Duhem processes. *J. Stat. Phys.* **96**, 415–427 (1999)

[25] Jarzynski, C.: Hamiltonian derivation of a detailed fluctuation theorem. *J. Stat. Phys.* **98**, 77–102 (2000)

[26] Jiang, D.Q., Qian, M. and Qian, M.P.: *Mathematical theory of nonequilibrium steady states - On the frontier of probability and dynamical systems.* (Lect. Notes Math. **1833**) Berlin: Springer-Verlag 2004

[27] Kolomeisky, A.B., Stukalin, E.B. and Popov, A.A.: Understanding mechanochemical coupling in kinesins using first-passage-time processes. *Phys. Rev. E* **71** 031902(8) (2005)

[28] Kou, S.C., Cherayil, B.J., Min W., English, B.P. and Xie, X.S.: Single-molecule michaelis-menten equations. *J. Phys. Chem. B* **109** 19068-19081 (2005)

[29] Lu, H.P., Xun, L. and Xie, X.S.: Single-molecule enzymatic dynamics. *Science* **282** 1877-1881 (1998)

[30] McQuarrie, D.A.: Stochastic approach to chemical kinetics. *J.Appl.Prob.* **4**(3), 413-478 (1967)

[31] Min, W., English, B. P., Luo, G., Cherayil, B.J., Kou, S.C. and Xie, X. S.: Fluctuating Enzymes: Lessons from Single-Molecule Studies. *Acc. Chem. Res.* **38** 923-931 (2005)

[32] Min, W., Gopich, I.V., English, B.P., Kou, S.C., Xie, X. S. and Szabo, A.: When Does the Michaelis-Menten Equation Hold for Fluctuating Enzymes. *J. Phys. Chem. B* **110** 20093-7 (2006).

[33] Murray, J.D: *Mathematical biology, 3rd Ed.* New York: Springer 2002

[34] Nicolis, G. and Prigogine, I.: *Self-organization in nonequilibrium systems: from dissipative structures to order through fluctuations.* New York: Wiley 1977

[35] Pury, P.A. and Caceres, M.O.: Mean first-passage and residence times of random walks on asymetric disordered chains. *J. Phys. A: Math. Gen.* **36**, 2695-2706 (2003)

[36] Qian, H.: Cycle kinetics, steady-state thermodynamics and motors–a paradigm for living matter physics. *J. Phys. Cond. Matt.* **17**, S3783-S3794 (2005)

[37] Qian, H.: Open-system nonequilibrium steady-state: statistical thermodynamics, fluctuations and chemical oscillations. *J. Phys. Chem. B.* **110** 15063-15074 (2006)

[38] Qian, H. and Elson E.L.: Single-molecule enzymology: stochastic Michaelis-Menten kinetics. *Biophysical chemistry* **101-102** 565-576 (2002)

[39] Qian, H., Saffarian S. and Elson E.L.: Concentration fluctuation in a mesoscopic ocillating chemical reaction system. *Proc. Natl. Acad. Sci. USA* **99** 10376–10381 (2002)

[40] Qian, H. and Wang H.: Continuous time random walks in closed and open single-molecule systems with microscopic reversibility. *Europhys. Lett.* **76**(1) 15-21 (2006)

[41] Qian, H. and Xie, X.S.: Generalized Haldane equation and fluctuation theorem in the steady-state cycle kinetics of single enzymes. *Phys. Rev. E* **74** 010902(4) (2006)

[42] Qian, H. and Reluga, T.C.: Nonequilibrium thermodynamics and nonlinear kinetics in a cellular signaling switch. *Phys. Rev. Lett.* **94** 028101 (2005)

[43] Qian, M.P. and Qian, M.: Circulation for recurrent Markov chains. *Z. Wahrsch. Verw. Gebiete* **59**, 203–210 (1982)

[44] Qian, M.P. and Qian, M.: The entropy production and reversibility of Markov processes. *Science Bulletin* **30**(3), 165–167 (1985)

[45] Qian, M.P., Qian, M. and Gong, G.L.: The reversibility and the entropy production of Markov processes. *Contemp. Math.* **118**, 255–261 (1991)

[46] Qian, M.P., Qian, C. and Qian, M.: Circulations of Markov chains with continuous time and the probability interpretation of some determinants. *Sci. Sinica (Series A)* **27**(5), 470–481 (1984)

[47] Van Kampen N.G.: *Stochastic Processes in Physics and Chemistry.* Amsterdam:North-Holland 1981

[48] Wang H. and Qian, H.: On detailed balance and reversibility of semi-Markov processes and single-molecule enzyme kinetics. *J. Math. Phys.* **48** 013303 (2007)

[49] Xie, X.S. and Lu, H.P.: Single-molecule enzymology. *J.Biol.Chem.* **274**(23), 15967–15970 (1999)

[50] Xie, X.S.: Single-molecule approach to enzymology. *Single Mol.* **2**(4), 229–236 (2001)

[51] Xie, X.S.: Single-molecule approach to dispersed kinetics and dynamic disorder: Probing conformational fluctuation and enzymatic dynamics. *J.Chem.Phys.* **117**(24), 11024–11032 (2002)

[52] Zhou, T.S., Chen, L.N., and Wang, R.Q.: A mechanism of synchronization in interacting multi-cell genetic systems. *Physica D* **211**, 107–127 (2005)