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Statistical determination of the step size of molecular motors

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Abstract

Molecular motors are enzymatic proteins that couple the consumption of chemical energy to mechanical displacement. In order to elucidate the translocation mechanisms of these enzymes, it is of fundamental importance to measure the physical step size. The step size can, in certain instances, be directly measured with single-molecule techniques; however, in the majority of cases individual steps are masked by noise. The step size can nevertheless be obtained from noisy single-molecule records through statistical methods. This analysis is analogous to determining the charge of the electron from current shot noise. We review methods for obtaining the step size based on analysing, in both the time and frequency domains, the variance in position from noisy single-molecule records of motor displacement. Additionally, we demonstrate how similar methods may be applied to measure the step size in bulk kinetic experiments.

1. Introduction

A large class of enzymes translocate, or 'step', along a linear protein or nucleic acid track by converting chemical energy to motion. Included in this class of enzymes are molecular motors in the kinesin and myosin families, and a host of processive nucleic acid enzymes, such as helicases, polymerases, exonucleases, type I endonucleases, and DNA translocases. For these enzymes, determining the unitary motion associated with a single biochemical cycle, i.e. the step size, is paramount for understanding the translocation mechanism of the protein along its track. More generally, the concept of a 'step' may be applied to any enzyme activity that results in quantized mechanical motion. This broader group includes F_1F_0 ATPase, which produces discrete rotations, and topoisomerases, which relax integer numbers of DNA supercoils. The

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reaction cycle of a motor protein can be viewed as a biochemical cycle, in which chemical energy is consumed, coupled to a mechanical cycle in which work is performed in advancing the protein one step. In this simple picture, the biochemical cycle is defined as a sequence of chemical and conformational states, such as ATP binding, hydrolysis, and product release, whereas the mechanical cycle is essentially defined by the distance the enzyme travels along its track in one cycle. An accurate measure of the step size therefore constrains possible mechanisms of translocation.

Measurements of the step size also provide insight into the coupling between the biochemical and mechanical cycles. This mechanochemical coupling can be thought of as a transmission that connects the chemical and mechanical cycles. The coupling ratio is the number of chemical cycles per step, which may be constant (tightly coupled), or variable (loosely coupled). These parameters correspond to the gear ratio and clutch slippage in the transmission analogy. On a structural level, the step size may provide insight into the enzyme domain motions required for translocation that in turn may serve as the basis for structural modelling.

The fundamental importance of the step size is underscored by the development of numerous methods for accurately determining this quantity for translocases. Sensitive single-molecule manipulation and measurement techniques, e.g. optical tweezers [1], magnetic tweezers [2], and single-molecule fluorescence detection [3], permit the direct observation of the discrete stepping of several molecular motors, in particular kinesin [4], dynein [5], and members of the myosin family [6–8]. These observations are possible as the relatively large step size of these proteins (∼5–35 nm) and long step times (at low ATP concentrations) afford large signal-to-noise ratios. Single-molecule measurements, in which varying loads opposing the mechanical step are applied, further permit an indirect measure of the step size through the load dependence of the enzymatic reaction rate [9]. For the particular case in which the mechanical transition is in rapid equilibrium, the change in enzymatic rate results from the additional work associated with translocating one step, δx, against the applied force, F [10, 11]. The forward rate, k, is therefore given by $k(F) = k_0^* \exp(-F \cdot \delta x / k_B T)$, where k_0 is the rate in the absence of force. Although step size determination is most easily accomplished employing single-molecule techniques, which avoid the averaging and dephasing inherent in bulk approaches, methods have been developed for indirectly determining the step size from bulk measurements. For example, using rapid stopped-flow kinetic measurement techniques, the number of ATP molecules hydrolysed per PcrA helicase as a function of DNA length has been determined, thereby permitting an indirect measurement of the PcrA helicase unwinding step size [12].

Lohman and co-workers developed a complex 'n-step' kinetic model for determining the step size of DNA helicases in bulk assays [13–15]. Pre-steady state kinetic measurements of the time course of the amount of DNA unwound exhibit a lag phase indicative of the number of rate-limiting steps required for unwinding a DNA molecule. A detailed n-step kinetic model including finite processivity and possible additional rate-limiting steps can be fitted to the time course of the appearance of unwound DNA. Global fits of this model to the data for unwinding of dsDNA of different lengths provide the enzyme step size [15]. Although this analysis was developed for the 'all or none' type of unwinding assay, in which only the appearance of the completely unwound DNA is monitored, it can be extended to other assays.

Bianco and co-workers [16] developed a bulk assay to measure the step size of RecBC helicase, which moves in the $3' \rightarrow 5'$ direction along DNA. They used a DNA template with a gap in the strand on which the RecBC translocates and monitored the unwinding of the DNA on both sides of the gap. The rationale is that the helicase can bridge a gap no larger than its step size. By varying the length of the double-stranded region before the gap and the length

of the gap, a step size of 23 nucleotides was suggested and a 'quantum inchworm' model was proposed for the motion of RecBC [16].

In this short review we are concerned with a powerful complementary approach for determining the step size of a processive enzyme, or, more generally, the fundamental unit of a repetitive biological process, through analysis of the variance of single-molecule time trajectories. We review methods of step size determination based on the analysis of the variance in both the time and frequency domains. Moreover, we generalize this single-molecule approach and propose a method for obtaining the step size from bulk measurements based on the analysis of the variance.

Analysis of noise has a long and illustrious history in both physics and biology. Einstein's theory of the random (Brownian) motion of pollen grains first described by Robert Brown provided one of the first connections between macroscopic observables and the microscopic atomic theory of matter [17]. Einstein's proposal, a version of what we now term the fluctuation dissipation theorem, permitted Perrin to make one of the first accurate determinations of Avogadro's number [18, 19]. In neuroscience, long before single-channel recordings were possible, Katz and Miledi [20] applied noise analysis to the problem of acetylcholine-mediated muscle end plate depolarization. By measuring the variance of the membrane potential they were able to deduce a quantal depolarization, which they attributed to the opening of individual ion channels. This analysis allowed them to make the first measurement of the depolarization potential of a single channel, as well as the number of channels involved in a single depolarization event. The advent of single-channel recording permitted direct measurements of these parameters, though noise analysis is still employed for channels that are not amenable to single-channel recordings, such as low conductance and difficult-to-isolate channels [21]. Analysis of variance carried out in this fashion permits the extraction of the fundamental quantum of a stochastic signal. Below, we present a detailed application of this principle for obtaining the step size measurement for a translocating protein.

The principle was introduced by Shottky in 1918 for deducing the charge of the electron from a statistical analysis of the current in a vacuum tube [22]. He derived the fluctuations in this current, which he called 'shot noise', by likening it to the noise of hail hitting a surface. The gist of the argument is as follows. The current I measured during a time interval t is due to a number $n(t)$ of electrons with charge e impinging on the anode: $I(t) = en(t)/t$. As the electrons are uncorrelated and arrive randomly on the anode, the probability of n electrons arriving during t, $P_t(n)$, is Poisson distributed:

$$P_t(n) = K^n \exp(-K)/n!$$

where K is the mean number of electrons that arrive during t. The average current is $\bar{I} = eK/t$ and its variance is $\langle (I - \bar{I})^2 \rangle = (e^2/t^2)\langle (n - K)^2 \rangle = e^2 K/t^2 = e\bar{I}/t$ (since the variance of a Poisson distribution is equal to its mean, K). The unit of charge, e, can therefore be deduced from the ratio of the average current and its fluctuations measured over a time interval t.

2. Time domain fluctuation analysis for the determination of the step size

Just as the variance in shot noise is proportional to the charge of the electron, the variance in the stepping records of a translocating protein is proportional to its step size. Block and co-workers first applied fluctuation analysis to single-molecule recordings of kinesin [23, 24]. Since the stepping of kinesin had been observed and characterized directly from time traces [4], their work focused on obtaining the number of rate-limiting steps in the kinesin biochemical cycle. We will follow a similar line of reasoning to obtain the step size from single-molecule recordings of enzyme displacement.

Consider a simple Poissonian molecular motor making one step of size d along its track (microtubule, actin, DNA, etc) in a mean time $\bar{\tau}$ (typically the average period of its ATP hydrolysis cycle). Since stepping is a random process, the number n of steps performed in a time t is Poisson distributed (like the shot noise):

$$P(n) = (t/\bar{\tau})^n \exp(-t/\bar{\tau})/n!.$$

The observed position $x(t)$ of the enzyme along its track after a time t is then

$$x(t) = nd + \eta(t) \tag{1}$$

where $\eta(t)$ is the positional Brownian noise, with mean $\langle\eta\rangle = 0$ and variance $\langle\eta^2\rangle = k_B T/\alpha$, and α is the combined spring constant of the measurement system (including the stiffness of the motor and the intrinsic stiffness of the optical or magnetic trap). The mean and variance of the displacement are given by

$$\langle x\rangle = d(t/\bar{\tau}) \tag{2}$$

$$\langle (x - \langle x\rangle)^2\rangle = d^2(t/\bar{\tau}) + \langle\eta^2\rangle. \tag{3}$$

From these expressions it is clear that the step size d can be obtained from the ratio of the time derivatives of the variance and the average position (i.e., the mean velocity); see figure 1.

If we now consider the more general case of an enzyme that makes one physical step at some point during an arbitrary biochemical cycle, the full cycle must be included in the analysis. For simplicity we consider only irreversible on-pathway transitions, although tools for calculating the completely general case have been developed [25]. As an example we consider a cycle with m states, during which a single step of displacement d is made:

$$1 \xrightarrow{k_1} 2 \xrightarrow{k_2} \cdots \xrightarrow{k_{m-1}} m \xrightarrow{k_m} 1.$$

The mean cycle time is given by

$$\bar{\tau} = \sum_{i=1}^{m} 1/k_i \tag{4}$$

and it has a variance

$$\sigma_\tau^2 = \langle(\tau - \bar{\tau})^2\rangle = \sum_{i=1}^{m} 1/k_i^2.$$

The randomness parameter, $r = \sigma_\tau^2/\bar{\tau}^2 = \sum_{i=1}^{m}(k_i\bar{\tau})^{-2}$, is indicative of the randomness of the enzymatic cycle (or stepping time) [25, 26]. If the biochemical cycle consists of a single transition ($m = 1$), or if one transition rate in the cycle is significantly slower than the others, $r = 1$. The time between steps, $P(\tau)$, is then exponentially distributed:

$$P(\tau) = \exp(-\tau/\bar{\tau})/\bar{\tau}.$$

Alternatively, if each of the m transitions has the same rate, $r = 1/m$, and the enzymatic cycle time is more narrowly distributed:

$$P(\tau) = m^m \tau^{m-1} \exp(-m\tau/\bar{\tau})/(m-1)!\bar{\tau}^m$$

(in the limit $m \to \infty$, this distribution becomes Gaussian with mean $\bar{\tau}$ and variance $\bar{\tau}^2/m$). In general r is inversely related to the number of rate-limiting steps in the enzymatic cycle.

The randomness of the enzymatic cycle affects the variance of the displacement of the translocating enzyme (i.e. more rate-limiting steps produces less randomness and a reduced variance). Thus while the mean velocity is still given by $v = \langle x\rangle/t = d/\bar{\tau}$, the variance in the displacement is [25]

$$\sigma_x^2 = \langle(x - \langle x\rangle)^2\rangle \approx d^2(t/\bar{\tau})r + \langle\eta^2\rangle. \tag{5}$$

The inclusion of additional steps in the biochemical cycle leads to an additional factor in the expression for the variance. The apparent step size determined by comparing the time

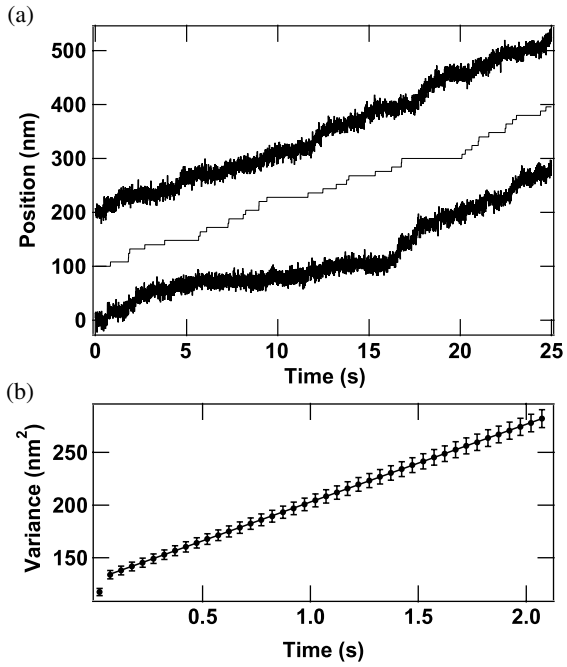

Figure 1. Determining step size through variance analysis in the time domain. (a) Simulations of single-molecule position recordings ($x(t)$) are generated by adding Gaussian noise to a stochastic stepping trace. Individual step transitions are masked by the noise. Two noisy traces are shown along with one trace without noise. Traces are displaced on the y axis for clarity. The average velocity for each trace is obtained by a line fit to $x(t)$, which is averaged over all traces to obtain the average run velocity v. For each trace the variance as a function of time is found using the average velocity v. These are then averaged together (b) and the slope of this average variance as a function of time divided by the velocity gives the step size. The simulated data had a step size of 8 nm, Gaussian noise with $\sigma = 8$ nm, and a stepping rate of 1.2 s^{-1}. Analysis was performed on 100 traces. The average velocity is 9.4 ± 0.2 nm s^{-1} and the slope of the average variance versus time trace is 73.84 ± 0.02 nm^2 s^{-1}, which combine to give a measured step size of 7.8 ± 0.3 nm.

derivative of the variance to the average velocity is the actual step size d multiplied by the randomness parameter r:

$$rd = \frac{1}{v}\frac{\partial \sigma_x^2}{\partial t}. \tag{6}$$

Therefore to accurately measure the step size d using this procedure, r must be measured independently. However, even if r is unknown, equation (6) will still yield a lower bound for the step size since, in the absence of backward steps, $rd < d$.

Whereas both the variance and average displacement grow linearly in time, the noise $\langle \eta^2 \rangle$ is constant; thus measurements of step size based on the variance are largely insensitive to noise. Indeed, the individual steps can be completely obscured by Brownian noise, yet the step size can still be recovered given sufficiently long time traces. By the same token, the instrument requires neither the spatial nor temporal resolution to capture individual steps. However, the duration of the measurement may be limited by the number of steps taken by the motor before it detaches from its substrate, i.e. its processivity. Consequently, variance based step size determination is only applicable to processive molecular motors. The principal limitation in this technique is the randomness factor that arises from the slowest transitions

in the biochemical cycle. If the details of the biochemical cycle are known, the randomness can be calculated, including the effects of off-pathway states, reversible reactions, and futile biochemical cycles [25]. Since this is rarely, if ever, the case, other means of determining the randomness are required. If one transition can be made rate-limiting, for example by working at limiting ATP concentration [27] or applying a significant load, the randomness will be close to one and the variance analysis will yield the step size. Conversely, if the step size is known, then measurements of r can reveal details of the biochemical cycle that may otherwise be difficult to obtain [27, 28]. In practice the time domain analysis of fluctuations is used almost exclusively to calculate r, rather than the step size.

3. Fluctuation analysis in the frequency domain for the determination of the step size

The fluctuations due to the stochastic stepping of an enzyme can also be analysed in the frequency domain to yield the step size, d. Variance analysis in the frequency domain has been used in a number of instances to determine the step size of processive enzymes, including UvrD helicase [29], the DNA translocase FtsK [30], and the unwinding step of topoisomerase IA [31]. Charvin and co-workers first developed the frequency domain approach to finding the step size [32] and we follow a similar derivation here.

We begin with the time-dependent position of an enzyme that has taken n steps of size d at various time points t_i $(i = 1, \ldots, n)$:

$$x(t) = d\sum_{i=1}^{n}\theta(t - t_i) + \eta(t) \tag{7}$$

where $\theta(t)$ is the Heaviside function, which is unity for positive t and zero otherwise, and $\eta(t)$ is the Brownian noise as previously defined. The Fourier transform of the position signal is given by

$$\tilde{x}(f) = \frac{d}{2\pi \mathrm{i} f}\sum_{i=1}^{n}\exp(2\pi \mathrm{i} f t_i) + \tilde{\eta}(f), \qquad f > 0. \tag{8}$$

For the case of a stochastic enzyme the step times are uncorrelated; hence the sum is over unit vectors pointing at random in the plane. The mean of the sum is zero but the variance is n. This allows us to compute the averaged power spectrum, $\langle S_x(f)\rangle$, of the fluctuations in the position measurement:

$$\langle S_x(f)\rangle = \frac{2\langle|\tilde{x}(f)|^2\rangle}{T} = \frac{d^2N}{2\pi^2 T f^2} + \langle S_\eta(f)\rangle = \frac{dv}{2\pi^2 f^2} + \langle S_\eta(f)\rangle, \qquad f > 0 \tag{9}$$

where N is the average number of steps in time T, $v = Nd/T$ is the mean velocity, and $\langle S_\eta(f)\rangle$ is the noise power spectrum of a particle undergoing Brownian motion in a harmonic potential with stiffness α,

$$\langle S_\eta(f)\rangle = \frac{k_\mathrm{B}T}{\pi\alpha f_0(1 + (f/f_0)^2)}, \tag{10}$$

where f_0 is the roll-off frequency of the noise (which decreases with increasing drag on the system). The important point is that at low frequencies ($f \ll f_0$) the power spectrum of the noise is flat (frequency independent), whereas the contribution of the stepping process to the fluctuations in position (the first term on the right in equation (9)) increases as $1/f^2$. For sufficiently long traces (i.e. sufficiently low frequencies in the power spectrum) the noise power arising from the random stepping will dominate the Brownian noise. The step size d can then be derived from a fit of the low frequency part of $\langle S_x(f)\rangle$ to the functional form: $dv/(2\pi^2 f^2)$; see figure 2.

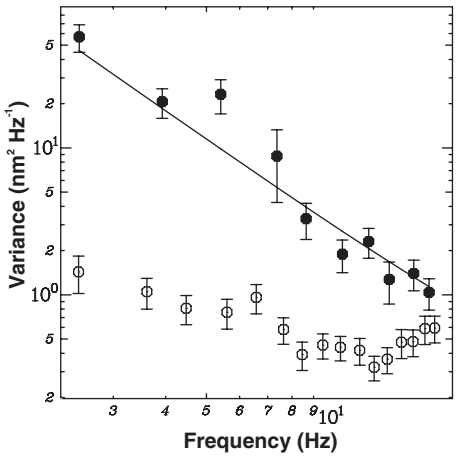

Figure 2. Determining step size through variance analysis in the frequency domain. Estimation of the step size of the DNA translocase FtsK in single-molecule experiments (figure taken from [31]). As an FtsK enzyme reels in the DNA at a rate v, $x(t)$ decreases. The quantized nature of the DNA translocation by FtsK results in an increase in the low frequency variance of $x(t)$. Compare the signal measured during FtsK activity (filled circles) with the noise signal (open circles) measured in the absence of FtsK activity. A fit of the low frequency components of the mean position variance $\langle S_x(f)\rangle$ to the functional form: $\langle S_x(f)\rangle = dv/(2\pi^2 f^2) + b$ (line), yields the enzymatic step size: $d = 12 \pm 2$ bp (assuming a single rate-limiting step).

In practice, the position fluctuations are obtained by subtracting a line of slope v, the average velocity, from the experimentally measured position trace. The average power spectrum of several such processed traces can be fitted to obtain the step size. The determination of step size is insensitive to the details of the method used to obtain the power spectrum; however, care should be taken to ensure that it is correctly normalized. This can be accomplished by analysing artificial data from simulations, or, preferably, from measurements of known trajectories generated by moving a piezoelectric stage, for example.

The frequency domain analysis of the step size is subject to the randomness of the step time distribution in the same manner as the time domain analysis, although the relation between the spectrum and the randomness parameter is less obvious. Charvin *et al* suggest how to compute the spectrum for an arbitrary reaction pathway [32]; however, we will not duplicate their arguments here. In general the same caveats and precautions concerning the time domain analysis also hold for the frequency domain analysis. In particular, note that the step size measured using these techniques is the physical step of the motor along its track, which does not necessarily correspond to the fundamental enzymatic step. For example, one could imagine a translocation mechanism in which the enzyme proceeds through several enzymatic turnovers before the protein changes position (i.e. a many to one coupling). The step size determined from the analysis of variance of single-molecule recordings would correspond to the physical motion of the protein, while the multiple enzymatic cycles required to produce one physical step would be reflected in the reduced randomness parameter r.

4. Variance analysis of bulk kinetic data to obtain the step size

In this final section we propose a method of determining the step size of a processive enzyme by analysing bulk kinetic measurements of the time required to move over a fixed distance. A typical example involves measuring helicase unwinding of dsDNA by quantifying the amount of completely unwound ssDNA as a function of time [33].

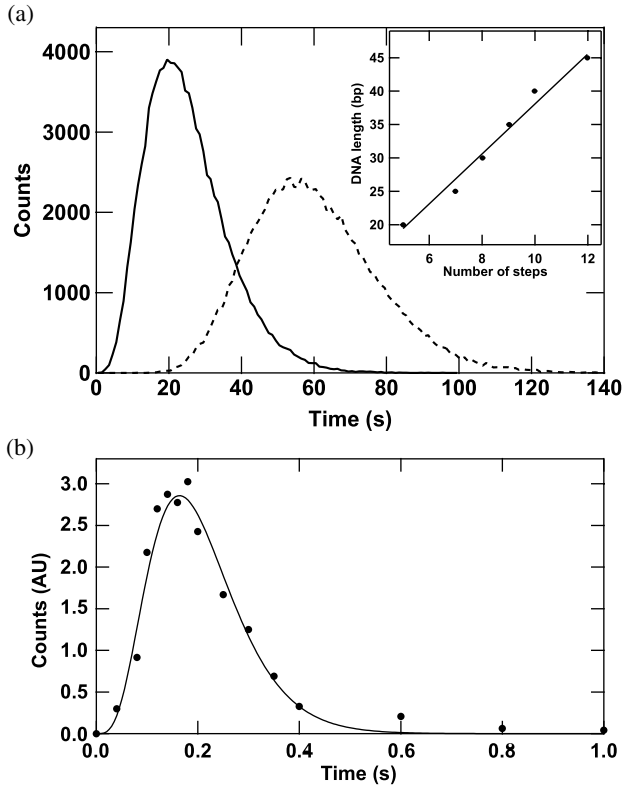

Figure 3. Variance based measurement of the step size from bulk measurements. (a) Results from a bulk DNA unwinding assay were simulated by averaging over 10^5 individual trajectories. The unwinding activity was modelled with a constant 4 bp step occurring at a single rate of 0.2 s^{-1}. Total unwinding was assumed when a number of bases equal to or greater than the DNA length had been unwound. The distribution of unwinding times is shown for dsDNA lengths of 20 bp (solid line) and 45 bp (dashed line). For the 20 bp DNA the variance in unwinding times is 126 s^2 and the average is 25 s. The mean time between steps τ derived from the ratio of the variance to the mean, $\tau = 5.04$ s, yields a step size of 3.97 bp. For the 45 bp DNA the calculated step size is 3.75 bp. The difference in step size calculated from the variance results from the extra step that occurs when the DNA length and the unwinding step are incommensurate. The actual step size can be determined by a line fit to the DNA length as a function of the calculated number of steps (inset). The slope of the line gives the actual unwinding step size. Fitting the results of the simulation for DNA lengths ranging from 25 to 45 bp gives a step size of 3.82 ± 0.03 bp, which compares well with the simulated step of 4 bp. (b) Fit to actual bulk data for DNA unwinding by UvrD helicase from Ali and Lohman [33]. The cumulative probability distribution for ssDNA ($l = 18$ bp) unwound by UvrD helicase was differentiated with respect to time to obtain the waiting time distribution of ssDNA formation. The waiting time distribution (filled circles) was fitted to the gamma distribution (equation (11); smooth curve), yielding a step time $\tau = 0.041 \pm 0.004$ s and number of steps $n = 4.9 \pm 0.4$, which give a step size of 3.6 ± 0.2 bp. For comparison, the mean (0.18 ± 0.03 s) and variance (0.009 ± 0.002 s^2) of the unwinding time were computed, which give a mean time between steps $\tau = 0.053 \pm 0.004$ s and a mean number of steps $n = 3.4 \pm 0.4$, corresponding to a step size $d = 5.3 \pm 0.4$ bp. These values compare reasonably well with those obtained from a global n-step kinetic model fit to the data from four different lengths of dsDNA [33]: $\tau = 0.054 \pm 0.004$ s and $\langle d \rangle = 4.4$ bp. The fit to the n-step model assumed a fixed integer number of steps n for each dsDNA length, which may account for the discrepancy between the values obtained using the different methods.

From these data we will show how to extract the number of base pairs opened per cycle d, by an analysis of the variance of the unwinding times t. The number of steps that a helicase

takes to reach the end of a length l of dsDNA is $n = l/d$. We assume that the time between steps follows an exponential distribution with mean time $\bar{\tau}$ and that there are no backward steps. We are interested in the distribution of the unwinding times, t, for a helicase to release an ssDNA of length l. For a sequence of n steps, the distribution of unwinding times, $F_w(t)$, is given by the gamma distribution:

$$F_w(t) = \frac{(t/\bar{\tau})^{n-1} \exp(-t/\bar{\tau})}{\bar{\tau} \cdot \Gamma(n)} \tag{11}$$

where $\Gamma(n)$ is the gamma function, defined for all positive values of n. If n is an integer, $\Gamma(n) = (n-1)!$. The mean and variance of the unwinding time for n steps are $\bar{t} = n\bar{\tau}$ and $\langle (t - \bar{t})^2 \rangle = n\bar{\tau}^2$. By measuring the rate at which ssDNA appears in solution (or the rate at which dsDNA disappears), the mean and variance of the unwinding time can be deduced and their ratio provides the mean time between steps $\bar{\tau}$. The number of steps is $n = \bar{t}/\bar{\tau}$ and the step size $d = l/n$; see figure 3. This approach is related to the n-state kinetic model discussed above [33]. Whereas the n-state model considers the *amount* of ssDNA produced as a function of time, the analysis of variance considers the *rate* of ssDNA production.

This simple example illustrates that the analysis of variance can equally well be applied to bulk kinetic measurements. Since bulk assays typically monitor the rate of product formation (or substrate depletion), the step size derived from the analysis of variance, or other methods, corresponds to the enzymatic step, which, as mentioned above, does not necessarily correspond to the physical step size. In this respect the two measurement techniques are complementary.

5. Conclusion

Variance analysis is a powerful statistical method for extracting features that are invisible in direct single-molecule or bulk data. It makes very few assumptions on the kinetics of the enzymatic motor, which is summed up in the randomness parameter. Under certain conditions this parameter can be estimated and the step size deduced. Alternatively, if the step size is known, the randomness parameter can be deduced from the variance analysis, yielding precious information on the number of rate-limiting steps in the enzymatic cycle. Variance analysis has been used in many different contexts to extract quantized information, such as the charge of the electron [22], the fractional charge of quasi-particles in the quantum Hall effect [34], single-ion channel depolarization events [21], the step size of molecular motors such as helicases [15, 29], and the number of supercoils relaxed by the action of topoisomerase 1A on DNA [31]. This simple and versatile method will certainly find new areas of application in the future.

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