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The asymptotic form of the probability density of sojourn times in randomly changing multistate systems

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Abstract

Recent investigations, motivated by applications to the interpretation of kinetic records of single molecules that randomly change their states, have shown that if the data is from a single state only, the asymptotic form of the probability density for the residence time in that state is Gaussian. In this paper we extend the formalism to show that when data is available from several states the joint density for any subset of them is a multivariate Gaussian. © 2000 Elsevier Science B.V. All rights reserved.

1. Introduction

A set of experimental techniques currently in wide use in biophysics, chemical physics, and physical chemistry, is that of single-molecule spectroscopy (hereafter referred to as SMS), [1–6]. This class of techniques enables an experimenter to study the kinetics of changes in state, either the result of an isomerization reaction or of a change in conformational state, of a single molecule rather than those of a large ensemble of molecules generated by bulk measurements. The latter can provide information about equilibrium properties, e.g., the values of equilibrium constants rather than those of individual rate constants. Some examples of systems whose kinetics are amenable to study by SMS techniques are the conformational fluctuations of a DNA molecule, one end of which is tethered to a surface [7], and similar fluctuations of single tRNA molecules on a surface [8].

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An example of the way that SMS can be implemented is by attaching fluorescent probes to the molecules being studied and measuring the subsequent decay of fluorescence as a function of time. If the experiment is carried out on an ensemble of many molecules this decay will be multiexponential, and the amplitudes of the exponentials will be proportional to the equilibrium populations in the different states. Therefore, this type of experiment can only provide information about the equilibrium state of the system. Since experimental techniques are now available to measure the kinetics of changes of state of single molecules it is possible to measure individual rate constants using SMS. One such technique is to periodically excite a single molecule using a laser, and record the history of changes of state during a finite measurement period of duration T. When the recording period is long enough so that many changes of state are observed, the fluorescence decay will again be multi-exponential, the coefficients of the exponentials being the random fraction of time, out of the total time T, spent by the molecule in each of the states [9,10].

In order to relate data from SMS measurements to the kinetic parameters it is necessary to have a theory that allows one to find the probability density for the fraction of time, out of the total observation period, T, that is spent by a molecule in each of the states. An exact theory for the SMS experiment has been provided only for the first-order isomerization reaction $S_1 \rightleftarrows S_2$, in [11]. The form of the results given there show that in the limit of very large T the required probability density is a Gaussian. This also is a result of the theory developed by Geva and Skinner [10] and can be found from an earlier, purely mathematical, work by Takacs [12]. The Gaussian limit is actually quite general, and is a consequence of the central-limit theorem. It also applies to more complicated Markovian [13] and non-Markovian reactions [14,15].

In Ref. [15], we have shown that when data is acquired from only a single state, some quite complicated reaction schemes could be reduced to an equivalent two-state non-Markovian scheme. This reduction, however, decreases the number of parameters, thus restricting the number of experimentally determinable parameters. As an example, to estimate all of the rate constants in the following reaction

$$A \underset{k_{BA}}{\overset{k_{AB}}{\rightleftarrows}} B \underset{k_{CB}}{\overset{k_{BC}}{\rightleftarrows}} C\,, \tag{1}$$

one needs to know the sojourn time densities for any two of the states. In this paper we envision a more complicated version of an SMS experiment in which the reaction scheme might encompass more than two states and in which estimates of the sojourn time densities are available from each of the states. It is obviously clear that data recorded from any individual pair of states can be used to provide estimates of all four rate constants in Eq. (1). However, if, in addition to the connectivity shown in that equation, one also allows for direct transitions between A and C, the added complication requires a more comprehensive theory along the lines discussed in the following exposition.

2. Description of the system

A standard starting point in analyzing SMS systems is to assume that fluorescence decay can be written as a multiexponential

$$\frac{I(t)}{I(0)} = \frac{1}{T}\sum_{j=1}^{n} t_j \mathrm{e}^{-k_j t} \tag{2}$$

in which t_j is the total time accumulated in separate sojourns in state j (hereafter referred to as S_j), T is the total time during which the system is observed, n is the number of discrete states, and the k_j are rate constants associated with the decay of the excited state S_j^* to S_j. As in our earlier work on the subject we assume that interconversions between excited states are either sufficiently slow so as to play a negligible role in determining system kinetics or may be absent entirely. We will be interested in finding the asymptotic form for the joint probability density of the t_j. The t_j are not independent since they satisfy the identity

$$\sum_{j=1}^{n} t_j = T\,. \tag{3}$$

However, it is reasonable to expect that the multivariate central-limit theorem can be invoked, for any subset, but not for the full set, of the n values of t. This allows one to say that the form of the joint probability density for any subset of the t_j at very long times should be a correlated Gaussian. We provide the formalism to calculate the variance–covariance matrix that defines the Gaussian. If desired, the formalism can also be extended so as to generate higher-order moments of the density.

A reaction can be regarded as a series of sojourns in each of the states available to the molecule. We will assume that a state can be either Markovian or non-Markovian, the latter suggestive of the existence of substates. A description of the reaction leading to Eq. (2) consists of two kinds of information. The first relates to the amount of time spent in a single sojourn in a given state, and the second relates to how the system states are connected. To describe the amount of time spent in a single sojourn in S_j we assign a probability density $\psi_j(t)$ such that the probability that a molecule spends a time between t and $t + \mathrm{d}t$ in a single sojourn in that state is equal to $\psi_j(t)\mathrm{d}t$. It will be assumed that at least the first two moments, $\langle t_j \rangle$ and $\langle t_j^2 \rangle$, are finite. We also make use of the probability that a single sojourn in S_j lasts for greater than t:

$$\Psi_j(t) = \int_t^{\infty} \psi_j(\xi)\,\mathrm{d}\xi\,. \tag{4}$$

The second type of information required for the analysis is a matrix of transition probabilities, $\mathbf{A} = (a_{mj})$, $j, m = 1, 2, \ldots, n$, in which a_{mj} is the probability that a sojourn in S_j is succeeded by one in S_m. One can always define the $\psi_j(t)$ in such a way that the diagonal elements a_{jj} are equal to zero. With this in mind we assert that for the two-state system the only possible form of $\mathbf{A}$ is

$$\mathbf{A} = \begin{pmatrix} 0 & 1 \\ 1 & 0 \end{pmatrix} \tag{5}$$

for the reaction $S_1 \rightleftarrows S_2$. More varied forms of $\mathbf{A}$ with zeroes on the diagonal are allowed when $n > 2$.

3. Mathematical formalism

In describing the behavior of the system just introduced we closely follow the formalism for such problems developed in Ref. [16]. At time T we let $\mathbf{t}=(t_1,t_2,\ldots,t_n)$ be a vector whose jth component, t_j, is the cumulative time spent in S_j during the period $(0,T)$. Let $p(\mathbf{t}|T)$ be the joint probability density for the times $\{t_j\}$ supplemented by the condition that the t_i sum to T. We will calculate exactly the joint Laplace transform

$$\hat{p}(\boldsymbol{\omega}|s) = \int_0^\infty \cdots \int_0^\infty \mathrm{e}^{-\omega\cdot\mathbf{t}}\,\mathrm{d}^n\mathbf{t} \int_0^\infty \mathrm{e}^{-sT} p(\mathbf{t}|T)\,\mathrm{d}T\,. \tag{6}$$

A knowledge of this transform allows us to calculate asymptotic properties of the moments.

Our calculation will be carried out in two stages. The first deals with the state at regeneration times, that is, the times at which there is transition between two states, or equivalently, the time at which a sojourn time in one of the states ends. Sets of linear integral equations will be derived for the functions that describe the evolution of the system.

Let $g_j(\boldsymbol{\tau},t)$ be the joint probability density for the time, t, spent in a single sojourn in S_j, and the amounts, $\tau_1,\tau_2,\ldots,\tau_n$, by which components of the vector $\boldsymbol{\tau}$ are to be incremented at the conclusion of that sojourn. By definition, $g_j(\boldsymbol{\tau},t)$ is

$$g_j(\boldsymbol{\tau},t) = \delta(\tau_1)\delta(\tau_2)\cdots\delta(\tau_{j-1})\delta(\tau_j - t)\delta(\tau_{j+1})\cdots\delta(\tau_n)\psi_j(t)\,. \tag{7}$$

By direct computation one sees that its Fourier–Laplace transform, defined as in Eq. (6), is expressed in terms of the Laplace transform of $\psi_j(t)$ as

$$\hat{g}_j(\boldsymbol{\omega},s) = \hat{\psi}_j(s+\omega_j)\,. \tag{8}$$

We will also need a second, related, function which is the joint probability-probability density for the increment added to $\boldsymbol{\tau}$ when the sojourn time is greater than t. This function will be denoted by $G_j(\boldsymbol{\tau},t)$. This can be found from Eq. (7) by replacing the function $\psi_j(t)$ in that definition by $\Psi_j(t)$. Its transform is, accordingly,

$$\hat{G}_j(\boldsymbol{\omega},s) = \frac{1-\hat{\psi}_j(s+\omega_j)}{s+\omega_j}\,. \tag{9}$$

Let $\rho_j(\mathbf{t},t)\mathrm{d}\mathbf{t},\mathrm{d}t$ be the joint probability that a sojourn in S_j ends during the interval $(t,t+\mathrm{d}t)$, the vector of accumulated residence times at that time lying in the volume $(\mathbf{t},\mathbf{t}+\mathrm{d}t)$. A set of integral equations will be derived for the joint probability densities

$\{\rho_j(\mathbf{t},t)\}$, $j=1,2,\ldots,n$. These functions will be used as an intermediate step in finding $p(\mathbf{t}|T)$ which is the probability density that describes $\mathbf{t}$ at the end of the time during which the system is observed. A function analogous to this will be denoted by $\rho_j^{(0)}(\boldsymbol{\tau},t)$ which is the joint probability density for the time at which the first sojourn ends, the first sojourn being in S_j and for the vector of accumulated times in each of the states to be equal to $\boldsymbol{\tau}$. It is necessary to use this function (although it clearly cannot contribute to asymptotic results) to account for the fact that the origin in time, $t=0$, may not coincide with the time of onset of a sojourn.

The integral equation satisfied by $\rho_j(\mathbf{t},t)$ is

$$\rho_j(\mathbf{t},t)=\theta_j\rho_j^{(0)}(\mathbf{t},t)+\sum_{l=1}^{n}a_{jl}\int_0^{t_1}\cdots\int_0^{t_n}\mathrm{d}^n\mathbf{t}'\int_0^{t}\rho_l(\mathbf{t}',\xi)g_j(\mathbf{t}-\mathbf{t}',t-\xi)\,\mathrm{d}\xi \tag{10}$$

in which θ_j is the probability that the system is in S_j at $t=0$. The first term on the right of this equation accounts for the possibility that the sojourn that ends at t is the first one, and the second term accounts for sojourns in $S_l(l\neq j)$ which end at an earlier time $\xi<t$ and are followed by a sojourn in S_j that lasts for the remaining time $t-\xi$. The joint transform of Eq. (10) is

$$\hat{\rho}_j(\boldsymbol{\omega},s)=\theta_j\hat{\rho}_j^{(0)}(\boldsymbol{\omega},s)+\sum_{l=1}^{n}a_{jl}\hat{\rho}_l(\boldsymbol{\omega},s)\hat{g}_j(\boldsymbol{\omega},s)\,. \tag{11}$$

A formal solution to this set of linear equations can be written formally as a ratio of determinants.

We denote by $p_j(\mathbf{t}|T)\mathrm{d}\mathbf{t}$ the probability that the sojourn times fall in the volume $(\mathbf{t},\mathbf{t}+\mathrm{d}\mathbf{t})$ when the total observation is equal to T. The $p_j(\mathbf{t}|T)$ are related to the $\{\rho_k(\mathbf{t}|t)\}$ by

$$p_j(\mathbf{t}|T)=\theta_jG_j^{(0)}(\mathbf{t}|T)+\sum_{l=1}^{n}a_{jl}\int_0^{t_1}\cdots\int_0^{t_n}\mathrm{d}^n\mathbf{t}'\int_0^{\infty}\rho_l(\mathbf{t}',\xi)G_j(\mathbf{t}-\mathbf{t}'|T-\xi)\,\mathrm{d}\xi \tag{12}$$

which shows that once that intermediate functions $\{\rho_k(\mathbf{t},t)\}$ are known, the passage to the $p_j(\mathbf{t}|T)$ is immediate. It will also be shown that, conditional on the existence of the first two moments of the $\psi_j(t)$, the asymptotic properties of the $p_j(\mathbf{t}|T)$ are directly expressible in terms of those of the $\rho_k(\mathbf{t}|T)$. Because of this we turn our attention to determining asymptotic properties of this set of functions.

4. Asymptotic properties

The vector of cumulative times spent in each state, $\mathbf{t}$, can be regarded as a sum of incremental times, which is equivalent to identifying it as an n-dimensional random walk. We now derive asymptotic properties of the functions $\{p_j(\mathbf{t}|T)\}$ in the asymptotic regime, defined by the property

$$T \gg \max_j \langle t_j \rangle \, . \tag{13}$$

When this inequality is satisfied the total time spent in each of the S_j will have been incremented many times. It is therefore natural to expect the asymptotic density for any strict subset of the vector $\mathbf{t}$ to be a correlated multivariate Gaussian. This intuitive notion can be proved in detail from properties of Eq. (11) in the limits $(\boldsymbol{\omega}, s) \to (\mathbf{0}, 0)$. The validity of this limiting form simplifies the analysis by requiring that we calculate moments and covariances up to second order.

Let $\hat{\boldsymbol{\rho}}(\boldsymbol{\omega}, s)$ be a vector whose jth component is $\theta_j \hat{\rho}_j(\boldsymbol{\omega}, s)$ and let $\boldsymbol{\rho}_0(\boldsymbol{\omega}, s)$ be a vector whose jth element is $\theta_j \hat{\rho}_j^{(0)}(\boldsymbol{\omega}, s)$. Further, let $\hat{\mathbf{m}}(\boldsymbol{\omega}, s)$ be a matrix whose jlth element is $a_{jl} \hat{g}_j(\boldsymbol{\omega}, s)$. The function $\hat{\boldsymbol{\rho}}(\boldsymbol{\omega}, s)$, the solution to Eq. (11) can be written as

$$\hat{\boldsymbol{\rho}}(\boldsymbol{\omega}, s) = [\mathbf{I} - \hat{\mathbf{m}}(\boldsymbol{\omega}, s)]^{-1} \boldsymbol{\rho}_0(\boldsymbol{\omega}, s) \, . \tag{14}$$

In similar fashion the joint transform of Eq. (12) can be rewritten in matrix form as

$$\hat{\mathbf{P}}(\boldsymbol{\omega}, s) = \hat{\mathbf{G}}_0(\boldsymbol{\omega}, s) + \hat{\mathbf{M}}(\boldsymbol{\omega}, s)[\mathbf{I} - \hat{\mathbf{m}}(\boldsymbol{\omega}, s)]^{-1} \boldsymbol{\rho}_0(\boldsymbol{\omega}, s) \, , \tag{15}$$

where $\hat{\mathbf{M}}(\boldsymbol{\omega}, s)$ is a matrix whose jlth element is $a_{jl} \hat{G}_j(\boldsymbol{\omega}, s)$. Neither $\hat{\mathbf{G}}_0(\boldsymbol{\omega}, s)$ nor $\boldsymbol{\rho}_0(\boldsymbol{\omega}, s)$ are singular in transform space, so that the only possible source of singularities are the ones in $[\mathbf{I} - \hat{\mathbf{m}}(\boldsymbol{\omega}, s)]^{-1}$. An examination of Eq. (14) shows that these singularities are found as the zeroes of the function

$$D(\boldsymbol{\omega}, s) = \det[\mathbf{I} - \hat{\mathbf{m}}(\boldsymbol{\omega}, s)]^{-1} = 1/\det[\mathbf{I} - \hat{\mathbf{m}}(\boldsymbol{\omega}, s)] \, . \tag{16}$$

The explicit form of the determinant is

$$D(\boldsymbol{\omega}, s) = \begin{vmatrix} 1 & -a_{12}\hat{g}_2 & -a_{13}\hat{g}_3 & \cdots & -a_{1n}\hat{g}_n \\ -a_{21}\hat{g}_1 & 1 & -a_{23}\hat{g}_3 & \cdots & -a_{2n}\hat{g}_n \\ \vdots & \vdots & \vdots & \vdots & \vdots \\ \vdots & \vdots & \vdots & \vdots & \vdots \\ -a_{n1}\hat{g}_1 & -a_{n2}\hat{g}_2 & \cdots & -a_{n,n-1}\hat{g}_{n-1} & 1 \end{vmatrix} \, . \tag{17}$$

From the fact that

$$\sum_{k=1}^{n} a_{kj} = 1 \tag{18}$$

and $\hat{\psi}_j(0) = 1$ it follows that when s is set equal to zero the sum of the elements of each column is equal to zero. This demonstrates that $D(\mathbf{0}, 0) = 0$. A further consequence of Gershgorin's theorem [17] is that $(\mathbf{0}, 0)$ is the only point at which $D(\boldsymbol{\omega}, s)$ vanishes. Because of this we expand $D(\boldsymbol{\omega}, s)$ around the origin, making use of the expression for $\hat{g}_j(\boldsymbol{\omega}, s)$ in Eq. (8). If we let $\varepsilon_j = s + \omega_j$ then we find that the expansion of $\hat{g}_j(\boldsymbol{\omega}, s)$ to second order in this parameter is

$$\hat{g}_j(\boldsymbol{\omega}, s) \approx 1 - \langle t_j \rangle \varepsilon_j + \frac{\langle t_j^2 \rangle}{2} \varepsilon_j^2 \, . \tag{19}$$

Hence, in a neighborhood of $(\mathbf{0},0)\,D(\boldsymbol{\omega},s)$ can be written as a quadratic form in the ε_j as

$$D(\boldsymbol{\omega},s) \approx \sum_{l=1}^{n} A_l\varepsilon_l + \frac{1}{2}\sum_{l=1}^{n}\sum_{m=1}^{n} B_{lm}\varepsilon_l\varepsilon_m \tag{20}$$

in which

$$A_l = \left.\frac{\partial D(\boldsymbol{\omega},s)}{\partial\varepsilon_l}\right|_{(\boldsymbol{\omega},s)=(\mathbf{0},0)} \quad \text{and} \quad B_{lm} = B_{ml} = \left.\frac{\partial D^2(\boldsymbol{\omega},s)}{\partial\varepsilon_l\partial\varepsilon_m}\right|_{(\boldsymbol{\omega},s)=(\mathbf{0},0)} . \tag{21}$$

In finding the coefficients in Eq. (20) we use the result that the derivative of a determinant is found by differentiating each column in turn, and setting $(\boldsymbol{\omega},s)=(\mathbf{0},0)$ [17]. Since, for example, the parameter ε_1 is found only in the first column of the determinant, finding an expression for A_1 only requires us to differentiate the first column in the determinant in Eq. (17) before setting the transform parameters equal to zero. In this way we find that

$$A_1 = \langle t_1\rangle \begin{vmatrix} 0 & -a_{12} & \cdots & -a_{1n} \\ a_{21} & 1 & \cdots & -a_{2n} \\ \vdots & \vdots & \vdots & \vdots \\ a_{n1} & -a_{n2} & \cdots & 1 \end{vmatrix} . \tag{22}$$

Similarly, the B_{lm} are found from Eq. (21) so that we can write

$$B_{11} = \langle t_1^2\rangle \begin{vmatrix} 0 & -a_{12} & \cdots & -a_{1n} \\ -a_{21} & 1 & \cdots & -a_{2n} \\ \vdots & \vdots & \vdots & \vdots \\ -a_{n1} & -a_{n2} & \cdots & 1 \end{vmatrix} \tag{23}$$

and

$$B_{12} = B_{21} = \langle t_1\rangle\langle t_2\rangle \begin{vmatrix} 0 & a_{12} & -a_{13} & \cdots & -a_{1n} \\ a_{21} & 0 & -a_{23} & \cdots & -a_{2n} \\ a_{31} & a_{32} & 1 & \cdots & -a_{3n} \\ \vdots & \vdots & \vdots & \vdots & \vdots \\ a_{n1} & a_{n2} & -a_{n3} & \cdots & 1 \end{vmatrix} . \tag{24}$$

The order of the determinants in the preceding expressions can always be reduced by one order by adding all of the rows to the first and taking advantage of the property in Eq. (18), so that, for example,

$$A_1 = \langle t_1\rangle \begin{vmatrix} 1 & 0 & \cdots & 0 \\ a_{21} & 1 & \cdots & -a_{2n} \\ \vdots & \vdots & \vdots & \vdots \\ a_{n1} & -a_{n2} & \cdots & 1 \end{vmatrix} = \langle t_1\rangle \left[\begin{matrix} 1 & \cdots & -a_{2n} \\ \vdots & \vdots & \vdots \\ a_{n2} & \cdots & 1 \end{matrix}\right| . \tag{25}$$

Since there are no restrictions on the $\langle t_j\rangle$ and $\langle t_j^2\rangle$ other than that they be finite, the results to be derived are not restricted to Markovian reaction schemes. This generalizes results in Ref. [12] which were found on the assumption that all reactions were first-order, or equivalently that they are Markovian systems.

The final expression for $\hat{p}(\boldsymbol{\omega}|s)$ in the neighborhood of the singular point can be expressed as

$$\hat{p}(\boldsymbol{\omega}|s) \approx \frac{N(\boldsymbol{\omega}, s)}{\sum_{l=1}^{n} A_l(s+\omega_l) + \frac{1}{2}\sum_{l=1}^{n}\sum_{m=1}^{n} B_{lm}(s+\omega_l)(s+\omega_m)}\,, \tag{26}$$

where the numerator is a non-singular function which does not contribute to asymptotic properties. The only property of this function required for subsequent calculations is that the approximation in Eq. (26) is properly normalized in the sense that $\hat{p}(\mathbf{0}|s) = 1/s$.

All that remains to find the corresponding relation in the time domain is to calculate the means, variances, and covariances from Eq. (26). This can be accomplished by first calculating the Laplace transforms of the moments by generating them from the Fourier transforms:

$$\langle \hat{t}_j(s) \rangle = -\left.\frac{\partial \hat{p}(\boldsymbol{\omega}|s)}{\partial \omega_j}\right|_{\omega=\mathbf{0}}, \quad \langle \hat{t}_j(s) \langle \hat{t}_k(s) \rangle = \left.\frac{\partial^2 \hat{p}(\boldsymbol{\omega}|s)}{\partial \omega_j \partial \omega_k}\right|_{\omega=\mathbf{0}}. \tag{27}$$

To simplify notation we define constants A, B and C_j, $j = 1, 2, \ldots, n$, by

$$A = \sum_{l=1}^{n} A_l, \qquad B = \sum_{l=1}^{n}\sum_{m=1}^{n} B_{lm}, \qquad C_j = \sum_{m=1}^{n} B_{jm}\,. \tag{28}$$

The lowest order term in the asymptotic expansion of $\langle t_j(T) \rangle$ is found, terms of the A's to be

$$\langle t_j(T) \rangle \underset{T\to\infty}{\approx} \frac{A_j}{A} T \tag{29}$$

plus a correction term that is O(1).

In order to express the covariances we define

$$U_j = \frac{C_j}{A_j} - \frac{B}{2A}\,. \tag{30}$$

In the same manner as was done in the calculation of the average in Eq. (29) we find that the lowest order term in the asymptotic expansion of the variances and co-variances are

$$\langle t_j(T) t_k(T) \rangle - \langle t_j(T) \rangle \langle t_k(T) \rangle \approx \left\{ \frac{A_j A_k}{A^2}(U_j + U_k) - \frac{B_{jk}}{A} \right\} T \tag{31}$$

with a correction term that is O(1). Thus, we can define an $n \times n$ variance–covariance matrix $\mathbf{V}(T) = \mathbf{V}_0 T$ in which the jkth entry in the constant matrix $\mathbf{V}_0$ is the term in brackets in the preceding equation.

A second function required to set the parameters of the Gaussian density is a vector $\boldsymbol{\mu}$, whose jth component is A_j/A, as follows from the coefficient of T in Eq. (29). Having this vector in hand, we can give an explicit expression for the asymptotic form of the multivariate Gaussian density for any subset of the t_i as [18]

$$p(\mathbf{t}|T) \approx \frac{1}{[2\pi T)^{n'} \det \overline{\mathbf{V}}_0]^{1/2}} \exp\left\{ -\frac{1}{2T}(\mathbf{t} - \boldsymbol{\mu} T)' \overline{\mathbf{V}}_0^{-1} (\mathbf{t} - \boldsymbol{\mu} T) \right\} \tag{32}$$

in which the reduced variance–covariance matrix, $\overline{\mathbf{V}}_0$, is found from $\mathbf{V}_0$ by deleting the contributions from all states not included in $\mathbf{t}$ and $n' < n$ is the number of states in the subset of interest. The fraction of time out of the total time T spent in S_j is $x_j = t_j/T$. The joint probability density for this set of variables will be denoted by $q(\mathbf{x}; T)$, which is given by

$$q(\mathbf{x}; T) \approx \sqrt{\frac{1}{\det\overline{\mathbf{V}}_0}\left(\frac{T}{2\pi}\right)^{n'}} \exp\left\{-\frac{T}{2}(\mathbf{x}-\boldsymbol{\mu})'\overline{\mathbf{V}}_0^{-1}(\mathbf{x}-\boldsymbol{\mu})\right\} . \tag{33}$$

This approaches a multivariate delta function $\delta(\mathbf{x}-\boldsymbol{\mu})$ as $T \to \infty$.

5. Discussion

The delta function limiting value may be understood in terms of the kinds of data obtained from measurements made in SMS experiments. If the states of one or more molecules are monitored for an infinite amount of time, then, by ergodicity the information obtained is equivalent to that available from measurements made over an infinite ensemble of molecules. The only information that such measurements provide relate to ratios of rate constants. Further information is available only by restricting the measurement times to be finite. To lowest order this corresponds to using the Gaussian approximation in Eq. (33). The variance−covariance matrix $\overline{\mathbf{V}}_0$ contains $n'(n'+1)/2$ further constants where $n' < n$ is the number of elements in the subset of the t_i whose properties are sought. Since the theory provides results expressed in terms of Fourier–Laplace transforms, it is, at least in theory, possible to generate information about higher moments. Higher order approximations to $q(\mathbf{x}; T)$ can then be found by expanding the joint density in an infinite series of Hermite polynomials. The coefficients in such an expansion are linear combinations of the moments.

As a final word we mention that the asymptotic Gaussian form of the probability density depends on the existence of a minimum of two moments for sojourn time densities. There is at least some experimental evidence in which the kinetics of the decay of an excited fluorophore differ from a single exponential and appear to have a long-tailed behavior [8,19]. For these systems the validity of using an asymptotic Gaussian density for cumulative sojourn times requires further investigation.

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