Nonequilibrium Response Spectroscopy and the Molecular Kinetics of Proteins

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Nonequilibrium response spectroscopy is a novel method of analyzing the molecular kinetics of voltage-sensitive proteins that involves measuring of the nonequilibrium response of the molecules to fluctuating fields with large amplitudes and frequencies. This response is exquisitely sensitive to features of the kinetics that are difficult or impossible to resolve by means of more traditional relaxation transient experiments. [S0031-9007(97)04963-6]

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As the sophistication of modern experimental techniques grows [1] biophysicists have started to seek an understanding and control of protein structure function that extends beyond mere heuristic descriptions [2] to the underlying physical mechanisms themselves. The study of voltage-gated ion channels, large transmembrane proteins in excitable cells that open a pore in response to electric fields, is an excellent example of this modern trend. Prospects for understanding and especially controlling these mechanisms ultimately rise or fall on a determination of the voltage dependence and underlying molecular mechanisms of channel gating, such as location of voltage sensors, electrical properties, and conformational substates. Ion channels exhibit their specific biological properties in living cells, and at temperatures above 273 K, where the gating mechanism is believed to involve complex rearrangements in the gross tertiary structure of the protein dominated by thermal activation on time scales of μs to minutes.

One of the main tools for studying and collating the electrophysiological properties of ion channels is the construction of Markov models [3] such as the one pictured in Fig. 1. The states of these kinetic models are often classified (as in Fig. 1) into closed (C), open (O), and inactivated (I) conformations, where the transition rates $\alpha_i(V)$ and $\beta_i(V)$ between these states are voltage dependent. Transitions between the states are thermally activated events and typically are assumed to take the exponential form $\alpha_i(V) = \alpha_i(0)e^{q_i\delta_i V/kT}$, and $\beta_I(V) = \beta_i(0)e^{-q_i(1-\delta_i)V/kT}$, which is in accord with Eyring rate theory, where V is the voltage applied across the cell membrane, $\alpha_i(0)$ and $\beta_i(0)$ are the activation rates at 0 mV, q_i are the gating charges, and $0 < \delta_i < 1$ are dimensionless parameters representing fractional electrical distances. These parameters determine a transition matrix $\tilde{\boldsymbol{W}}$ for a discrete master equation $\dot{\vec{\boldsymbol{\rho}}}(t) = \tilde{\boldsymbol{W}}[V]\vec{\boldsymbol{\rho}}$ that describes the evolution of the probability distribution vector $\vec{\boldsymbol{\rho}}(t)$ over the n conformational states of the model. If such models are good ones, the states and transition rates give information about the gross molecular conformations and electrical properties of the ion channel protein. They are falsifiable hypotheses that suggest new experiments, and are *representations of the kinetic information obtained experimentally,* but should not be construed as unique representations of the actual molecular motions.

The voltage clamp technique, in which the voltage across a cell membrane is controlled by a feedback circuit [4] that balances (and therefore measures) the net current, has been the best biophysical tool for the study of voltage-sensitive channels for almost half a century. Since its initial development [5], a basic set of voltage clamp protocols and the ideas behind them have dominated electrophysiological studies of the properties of ion channels. These protocols are based on potential stepping, the situation in which the voltage across a membrane is stepped from a holding potential to a test potential, or at a few points in time, and the current transient recorded. Information about the electrophysiological properties of a channel is then obtained from an analysis of these relaxation transients since the relaxation of the probability distribution can be calculated formally from the master equation, $\vec{\boldsymbol{\rho}}(t) = \exp(t\tilde{\boldsymbol{W}}[V])\vec{\boldsymbol{\rho}}(0)$, where $\vec{\boldsymbol{\rho}}(0)$ is the initial distribution when the voltage is stepped to V at $t = 0$. While the basis for much of electrophysiology, the relaxation transient method also sets some limitations since it is generally recognized that very different kinetic models can lead to very similar current transients when the voltage is held constant [6].

We first present a generic analysis of such experiments that reveals the source of their limitations. We wish to determine the elementary voltage-dependent rates of transition between the n gross conformational states where in this case there are *no means to independently determine these quantities,* a situation that determines both the

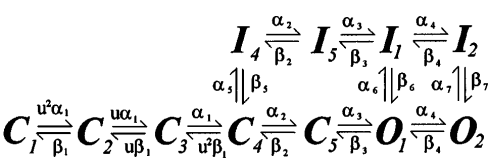

FIG. 1. Our model of the human cardiac sodium channel gating. The voltage dependence of the rates is described in the text, and u is a dimensionless parameter.

character and the universality of the experimental problem. While here we explicitly discuss voltage clamp experiments with ion channels, analogous limitations arise in the analysis of the decay of a correlation function in any complex system. A measurement of transition rates requires ensemble measurements. The state $\vec{\boldsymbol{\rho}}$ of an ensemble of channels is a point $(n-1)$-dimensional space (normalization requires $\sum_i \rho_i = 1$), which we will call the *kinetic manifold* of the channel. Current recordings are measurements that determine only certain projections of the distribution vector—$(n-1)$-to-1 mappings from the kinetic manifold to the observable current that are fixed by the physics of the molecule. The macroscopic ionic current (the quantity measured below) is equivalent to a measurement of the mean probability of the conducting (open) state or states $I = \sum_i g_{O_i} \rho_{O_i}$. Single channel measurements can distinguish between states with different conductances g_{O_i}. Gating currents (displacement currents that result from movements of the gating charges) are measurements of the rate of change of the total gating charge $\dot{C}(t) \propto \vec{\boldsymbol{Q}} \cdot \dot{\vec{\boldsymbol{\rho}}}(t)$, where $Q_i = \sum_{j=1}^{i} q_j$.

The maximum amount of information can be extracted from a given data type by measuring the quantity on all possible ensemble states $\vec{\boldsymbol{\rho}}$, a situation that is also impossible since we cannot prepare an arbitrary ensemble state. The easiest distributions to prepare lie on the one-dimensional *equilibrium submanifold* (parametrized by V) of equilibrium states, $\vec{\boldsymbol{\rho}}_{\mathrm{eq}}(V)$ (where $\tilde{\boldsymbol{W}}[V]\vec{\boldsymbol{\rho}}_{\mathrm{eq}}(V) = 0$), by allowing the channels to relax at a fixed potential V. Further distributions can be prepared from this set of states by stepping (at $t = 0$) to the new voltage V'. The channel ensemble executes a brief excursion away from the equilibrium submanifold before returning to it at the new point $\vec{\boldsymbol{\rho}}_{\mathrm{eq}}(V')$. While this excursion is taking place the channel explores a one-dimensional trajectory, $\vec{\boldsymbol{\rho}}_1(V, V', t) = \exp(t\tilde{\boldsymbol{W}}[V'])\vec{\boldsymbol{\rho}}_{\mathrm{eq}}(V)$. Since the points reached by stepped potential experiments are parametrized by not more than three parameters (V_0, V', and t), when $n - 1 \gg 3$, the set of all possible distributions reachable by stepped potential experiments (the *one-step submanifold*) is one of very large codimension with respect to the whole kinetic manifold. In other words, one-step experiments hardly explore the kinetic phase space at all—the source of a great deal of ambiguity. Models based on data from these experiments are corresponding ambiguous.

Standard relaxation transient experiments thus involve two independent limitations: one due to observational (projection) limitations and the other due to limitations of protocol. While a great deal of work involving ion channels has gone into removing the limitations of projection by developing new types of measurements, little work has been done to resolve limitations of standard protocols, which are so fixed in the experimental repertoire that it is often difficult to do anything substantially different with the commercial data-gathering software programs commonly used.

Since protocol ambiguities arise from limitations in our ability to experimentally explore all points in the kinetic phase space, they can be removed by enlarging the region of kinetic phase space that is explored by driving the channels off the one-step manifold. For instance, if the changes are rapid enough, the two-step manifold will have a dimensionality of no more than 5 (parametrized by V, V', V'', t, and t'). The logical extension of this idea is to change the voltage very rapidly at a large number of points in time, a method we call *nonequilibrium response spectroscopy* (NRS). In order to drive the channel ensemble off the one-step manifold the voltage must fluctuate more rapidly than the channel relaxes to equilibrium. If the voltage fluctuations are statistically stationary the ensemble will be driven toward a nonequilibrium stationary distribution. This is the notion of "nonequilibrium" we have in mind here, as distinguished from mere relaxation.

We used the model shown in Fig. 1 to fit data from macroscopic ionic current recordings from human heart sodium channels (hH1a) heterologously expressed in HEK293 cells [7]. Ionic currents of the model in response to a step in voltage are given by $I(t) = g(V)(V - V_r)\vec{\mathbf{e}}_O \cdot \vec{\boldsymbol{\rho}}(t)$, where V_r is the reversal potential, $\vec{\mathbf{e}}_0$ is the projection vector used to extract the probability of the open (conducting) states from $\vec{\boldsymbol{\rho}}(t)$, and $g(V)$ is the instantaneous conductance that takes into account voltage dependencies of the conductance that are independent of gating, including rectification due to ionic concentration differences across the membrane and voltage-dependent block by divalent cations. The reversal potential and $g(V)$ are determined independently by experiment [7] by standard techniques. Our model is similar to one proposed for the sodium channels of the squid giant axon [8], except that it has the additional open and inactivated states O_2 and I_2. The model in [8] was based on combined information from single channel, macroscopic ionic, and gating current recordings, and reproduced all of these types of data very well. It thus represents the state of the art with respect to modeling sodium channels. While some differences are to be expected between the cardiac and neuronal isoforms, there are major similarities because of the close homology between them. Model parameters [9] were determined by minimizing the chi-squared error between the set of 10 stepped potential relaxation transients and the model predictions for these transients [Figs. 2(a) and 2(b)] [7]. The results were in good agreement with the findings of many other researchers that used data from stepped potential series.

The homogeneous form of the kinetic equation can be preserved at the expense of allowing the voltage itself to fluctuate in a random but Markovian way. The simplest example of this type of voltage noise is telegraph noise [10]: a stochastic process V_t that switches at random times between two states, $V_t \in \{V_+, V_-\}$, at the mean rate $\omega/2$. The time-dependent transition rates in the model are then given adiabatically by $\alpha_i(t) = \alpha_i[V(t)]$, $\beta_i(t) = \beta_i[V(t)]$, an approximation that remains valid so long as the intraconformational fluctuation frequencies

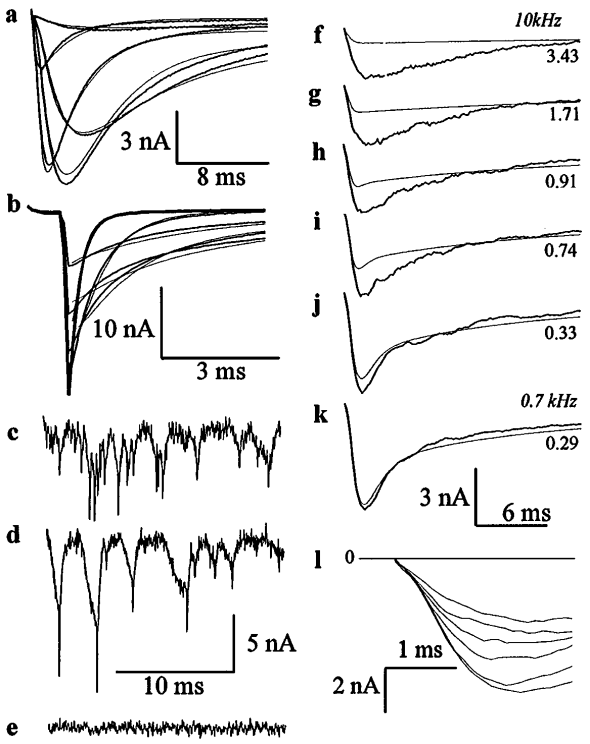

FIG. 2. Experiment showing NRS data is more sensitive than standard relaxation transient data. Model transients were calculated from the analytic expressions in the text using subroutines from Matlab. The reversal potential was 39.1 mv. All data from the same cell except for (d), and carried out at 282 K. (a),(b) Relaxation transients (heavy lines) and model predictions (thin lines) for steps from a holding potential of -150 mV to (a) -76, -58, -46, -22, 14 mV (activation) and (b) to 30 mV for 1 ms, and then to -120, -90, -66, -42, -12 mV (deactivation). The model was based on an optimal fit to the data shown in (a) and (b). (c)–(e) Raw current traces in response to voltage fluctuations with $V_+ = -30$ mV and $V_- = -120$ mV, and with bandwidths of (c),(e) 10 kHz and (d) 2 kHz. (d) Control data from untransfected cell with no sodium channels. (f)–(k) Model predictions (thin lines) and NRS relaxation transients (heavy lines) formed by averaging 500 raw data traces such as (c) and (d). Fluctuation band widths (in kHz) (f) 10, (g) 5, (h) 3, (i) 2, (j) 1, (k) 0.7. (l) Superposition of first few *ms* of (f)–(k) showing the decrease of the rise rate with increasing frequency.

($\simeq$80 kHz) [11] are much larger than the bandwidth ω of the voltage fluctuations ($\leq$10 kHz in the experiments described below) [7]. However, when the bandwidth of the voltage fluctuations is equivalent or greater than some or all of the *interconformational* transitions between states, the response of the channel can become very sensitive to a subtle feature of the underlying kinetics.

An n-state channel described by a kinetic scheme such as pictured in Fig. 1 and driven by the telegraph noise voltage fluctuations can be described by the $2n$-dimensional conditional master equation $\dot{\vec{\mathcal{P}}} = \tilde{\boldsymbol{\mathcal{W}}}\vec{\mathcal{P}}$, where

$$\tilde{\boldsymbol{\mathcal{W}}} = \begin{pmatrix} \tilde{\boldsymbol{W}}[V_+] - (\omega/2)\mathbb{1} & (\omega/2)\mathbb{1} \\ (\omega/2)\mathbb{1} & \tilde{\boldsymbol{W}}[V_-] - (\omega/2)\mathbb{1} \end{pmatrix}, \quad (1)$$

and where $\vec{\mathcal{P}} = (\vec{\boldsymbol{\rho}}_+, \vec{\boldsymbol{\rho}}_-)$ is a $2n$-dimensional conditional probability distribution vector, where $\vec{\boldsymbol{\rho}}_\pm$ is the conditional probability vectors *given that* the voltage is in the $V_\pm$ states. It may help to think of the system as an extended Markov model with states specified by both channel and voltage indices. The formal solution for the evolution of the conditional probability vector is $\vec{\mathcal{P}}(t) = \exp(t\tilde{\boldsymbol{\mathcal{W}}})\vec{\mathcal{P}}(0)$, where $\vec{\mathcal{P}}(0)$ is determined by the initial state of both the channel and the voltage. In all the experiments done here the initial state of the voltage was set to V_+, so $\vec{\mathcal{P}}(0) = (\vec{\boldsymbol{\rho}}(0), \vec{\mathbf{0}})$. The simplest way to use this method to analyze whole-cell recordings is to compute averages over many different realizations of the individual fluctuating current traces in response to pulses of telegraph noise of a given duration. The mean transient whole-cell current is $\langle I\rangle = \langle I_+\rangle + \langle I_-\rangle$, where $\langle I_\pm\rangle = g(V_\pm)(V - V_\pm)\vec{\mathbf{E}}_O^{\pm} \cdot \vec{\mathcal{P}}(t)$, and where $\vec{\mathbf{E}}_O^{+} = (\vec{\mathbf{e}}_O, \vec{\mathbf{0}})$ and $\vec{\mathbf{E}}_O^{-} = (\vec{\mathbf{0}}, \vec{\mathbf{e}}_O)$. Information is also contained in the fluctuations of the individual current traces but is not very helpful from the standpoint of accurate kinetic analysis because, in contrast to the average current transients, only the one-dimensional stationary case corresponding to the outdated and quantitatively incorrect Hodgkin-Huxley model is amenable to a simple mathematical treatment [10].

Figures 2(f)–2(m) show mean transient responses to telegraph noise with bandwidths that vary from 700 Hz to 10 kHz together with the corresponding predictions of our model. The experiment is designed so that the input bandwidth is high enough that its effect on the response is negligible. We are able to achieve such high frequency voltage control by making use of a specially designed low resistance pipette as discussed in more detail in [7]. The resistance of the pipettes in solution can be as low at 100 kΩ. Similar techniques have been used in our lab for a number of years [12], and under certain conditions when series resistance compensation is used RC times of as little as 5 μs (corresponding to a corner frequency of 30 kHz) can be achieved. Here such high speeds were not required. The data shown were obtained with an input bandwidth just in excess of 10 kHz, and with series resistance compensation, since this was all that was necessary to focus on the most interesting effects which were in the range between 2 and 6 kHz, well below our corner frequency.

Our model was based on stepped potential data and for low frequencies continued to prove a good predictor of the mean current in line with the analysis presented above. Since we used a fairly complete set of activation and tail current recordings that spanned the range of physiological potentials, the model represents nearly the best that can be done with a stepped potential series, and model parameters were, in fact, consistent with the finding of other researchers. For higher frequencies a mismatch begins to occur at frequencies of about 1–2 kHz. This is an indication of the discrepancy between the model and nature, and therefore represents information invisible to relaxation transient analysis [Figs. 2(a)–2(b)]. After the

analysis presented above it should not be surprising that this mismatch starts to occur in the region of most rapid change in the amplitude of the response, which nearly doubles when the frequency is lowered from 3 to 1 kHz. Over much higher and lower frequencies the peak inward currents change very little.

The response saturates above 6–10 kHz, an effect that occurs when the correlation time of the fluctuating voltage is much less than the relaxation time of the channel. Note that this frequency is well below the input corner frequency, and is just an expression of the highest frequencies of the channel kinetics. This case can be treated by replacing the inhomogenous kinetic equation $\dot{\vec{\rho}}(t) = \tilde{\boldsymbol{W}}[V(t)]\vec{\rho}$ by the homogenous one, $\dot{\vec{\rho}}(t) = \langle\tilde{\boldsymbol{W}}\rangle_V\vec{\rho}$, where $\langle\tilde{\boldsymbol{W}}\rangle_V = \int dV\,\rho_0(V)\tilde{\boldsymbol{W}}[V]$, where $\rho_0(V)$ is the stationary probability distribution of the voltage fluctuations. The response will be independent of all the features of the noise except the stationary probability density and therefore tractably analyzed for any kind of noise that has a sufficiently short correlation time. The model does not show the same saturation frequency as the data, another sign of the model's shortcomings that cannot be seen by standard experimental techniques.

Attempts were made to optimize the model parameters by including NRS data from Figs. 2(f)–2(m) in the optimization set with the stepped potential data. We found that no model of the form shown in Fig. 1 can simultaneously reproduce all the data in Fig. 2. Thus the information obtained from NRS forces us to consider a rather different kind of kinetic scheme than the one shown in Fig. 1. The ability of NRS to falsify models that cannot be falsified with current techniques should provide a powerful new tool for increasing our understanding of the kinetics of voltage-sensitive proteins. The need to falsify degenerate models has been and continues to be behind the search for new and different ways to measure channel gating.

There is an important difference in the symmetry properties of the operators $\tilde{\boldsymbol{W}}$ and $\tilde{\boldsymbol{\mathcal{W}}}$ that lies at the root of the sensitivity of this method: $\tilde{\boldsymbol{W}}$ is the evolution operator of a microscopically reversible system, whereas $\tilde{\boldsymbol{\mathcal{W}}}$ is not. When there are "loops" in the kinetic diagram (as is the case here) the latter will evolve to a stationary distribution that lacks detailed balance. As is beginning to be understood, microscopically irreversible systems are capable of exhibiting many types of unusual behavior that exhibit very sensitive, even singular [10,13] dependence on specific details of the kinetics. The data in Figs. 2(e)–2(m) are just one example of this sensitivity. With simple but general arguments Landauer has emphasized a similar point for a number of years [14]. As far as we are aware this is the first time this idea has been used as an experimental tool for a more sensitive determination of the underlying kinetics of a system.

The master equation appears in many contexts in physics, chemistry, and biology, and analogous methods could be introduced to support model selection and kinetic analysis in these other contexts. We believe these ideas should be of interest not only to experimental researchers studying protein dynamics, but also those studying statistical and nonlinear physics because they show theoretical ideas from these fields can have direct practical application to experimental biophysics. Our experimental technique also provides an arena in which to explore nonequilibrium fluctuation-induced phenomena, and is therefore of interest on purely physical grounds.

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