Current Noise Reveals Protonation Kinetics and Number of Ionizable Sites in an Open Protein Ion Channel

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In analogy to current fluctuations found in solid state electronic microstructure devices, excess noise generated by the reversible ionization of sites in a transmembrane ionic channel was observed. By analyzing the *p*H-dependent fluctuations in the current through fully open single channels formed by the α-toxin protein, we were able to evaluate the protonation rate constants, the number of sites participating in the protonation process, and the effect of recharging a single site on the channel conductance.

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Ions in aqueous solution cannot cross the poorly polarizable lipid bilayer membranes surrounding cells [1]. Instead, they pass through ion channels formed by a class of membrane spanning proteins that play key roles in the generation of nerve action potentials, cell-cell communication, and other critical cellular functions [2]. Protein ion channels have multiple conformation states, some of which have a hydrophilic pore open to the flow of ions. The difference in energy between these states is sufficiently small that thermal fluctuations cause ion channels to switch between different levels of conductance. Transitions between states in a single molecule can therefore be observed as a fluctuating ion current [3] in analogy to recent observations of the light-scattering signal from a single trapped ion switching between different energy levels [4,5].

Noise analysis has been successfully used to develop a molecular understanding of physical and chemical systems [6]. In biological systems, shot noise and noise from conformational variations are now widely recognized as sources of fluctuations in open channel currents [7].

In this study, we demonstrate that noise analysis can also be used to measure the rate constants of rapid chemical reactions that occur within the pore of a channel, if those reactions modulate the open channel conductance. We report that current noise may be produced by the reversible protonation of amino-acid sites in the channel-forming molecule. Moreover, we show that this technique can be used to determine the number of ionizable sites that modulate the channel conductance. The mechanism of the phenomenon we report here is analogous to that found for conductance fluctuations in solid state devices where recharging of a single trap [8,9] or a chain of localized states [10] was shown to be the fluctuation source.

Current through the channel formed by the α-toxin protein [11] was measured with Ag-AgCl electrodes placed in compartments of a Teflon chamber illustrated in Fig. 1. The current recordings in Fig. 2 show the spontaneous formation of a single channel at three different *p*H values. Two features are clearly seen. First, the mean conductance of the α-toxin channel decreases when the *p*H is increased over the range $4.5 \leq pH \leq 7.5$, in good agreement with results reported earlier [12,13]. Second, there is a difference in the current noise of the channel's open state at the three different *p*H values. The noise track, corresponding to current through the channel at *p*H 5.8, is wider compared to those at *p*H 4.5 and *p*H 7.5, showing the nonmonotonic variation of the open channel noise with *p*H.

The spectral density at $pH \approx 6$ is significantly larger than the shot-noise anticipated for these currents. Figure 3(a) illustrates this for the open channel current of 1.0×10^{-10} A at *p*H 5.9 (top trace). The magnitude of noise averaged in the bandwidth 200–2000 Hz with the background subtracted is illustrated in Fig. 4.

A simple model based on a first-order reversible protonation reaction accounts for these results. Specifically,

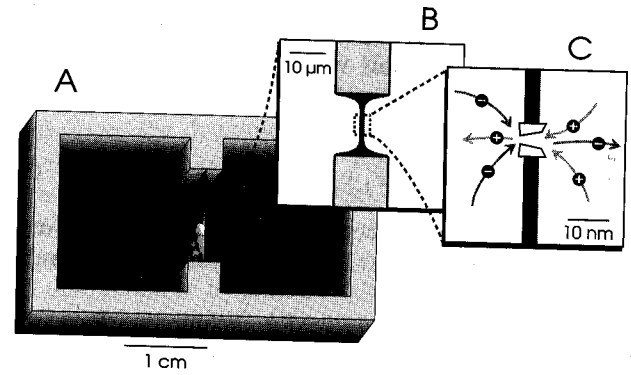

FIG. 1. Schematic diagram of the sample chamber (*A*), partition with a lipid bilayer membrane (*B*), and a transmembrane protein ion channel (*C*). Solvent-free diphytanoyl phosphatidylcholine membranes were formed on a ~50 μm diam hole in a 15 μm thick Teflon partition [13].

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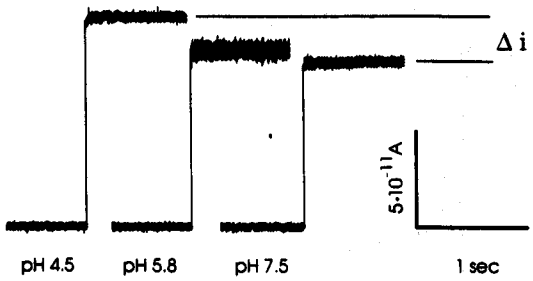

FIG. 2. Recordings of the current for three different values of pH, filtered at 2 kHz. The stepwise increases in the currents correspond to the spontaneous formation of a channel. In a typical experiment with $1M$ NaCl solutions, a single channel appeared as a 10^{-10} A current jump with a lifetime of tens of seconds, after which it usually switched to a much lower conducting state. We restrict this study to the transport characteristics of the fully open channel. Unless otherwise stated, the aqueous solutions contained $1M$ NaCl, 2.5 mM MES (monohydrate ethanesulfonic acid), $T=(24.0\pm1.5)°$ C, and the applied voltage was 150 mV, positive at the side of protein addition.

the effect of varying the pH on the noise spectral density and conductance of single open α-toxin channels can be described assuming the pore can access two distinct states of ionization, W_1 and W_2 (see Fig. 5), which differ in pore conductance:

$$W_1+\mathrm{H}^+\underset{k_D}{\overset{k_R}{\rightleftharpoons}}W_2, \tag{1}$$

where k_R ($M^{-1}\mathrm{s}^{-1}$) and k_D (s^{-1}) are the rate constants for the association and dissociation of a proton with an ionizable site on the channel, respectively, and W_2 is the state with increased conductance.

The probabilities, p_1 and p_2, that a channel occupies state W_1 or W_2 are given by

$$p_1=\tau_1/(\tau_1+\tau_2)=1/(1+10^{p\mathrm{K}-p\mathrm{H}}),\quad p_2=1-p_1, \tag{2}$$

where τ_1 and τ_2 are the mean times spent in states W_1 and W_2, respectively, $\tau_2=1/k_D$ and $p\mathrm{K}\equiv\log(k_R/k_D)$. For this process, we can write the spectral density of current noise $S_{i,H}(f)$ using Machlup's original derivation for random telegraph signals [14]:

$$S_{i,H}(f)=4(\Delta i_{1,2})^2\tau^2/\{(\tau_1+\tau_2)[1+(2\pi f\tau)^2]\}, \tag{3}$$

where $\Delta i_{1,2}$ is the difference in current (in A) between states W_1 and W_2, $\tau\equiv\tau_1\tau_2/(\tau_1+\tau_2)$ (in s), and f is the frequency (in Hz).

The model may be generalized to include the effect of n identical independently ionizable sites, which could correspond either to a channel comprised of an oligomer [15] or a monomer with n titratable sites, that modulate the conductance. Contributions to the spectral density from n independent sites add, increasing the total value by a factor of n. On the other hand, $\Delta i_{1,2}$ in Eq. (3) should then be replaced by $\Delta i/n$, where Δi is the difference in

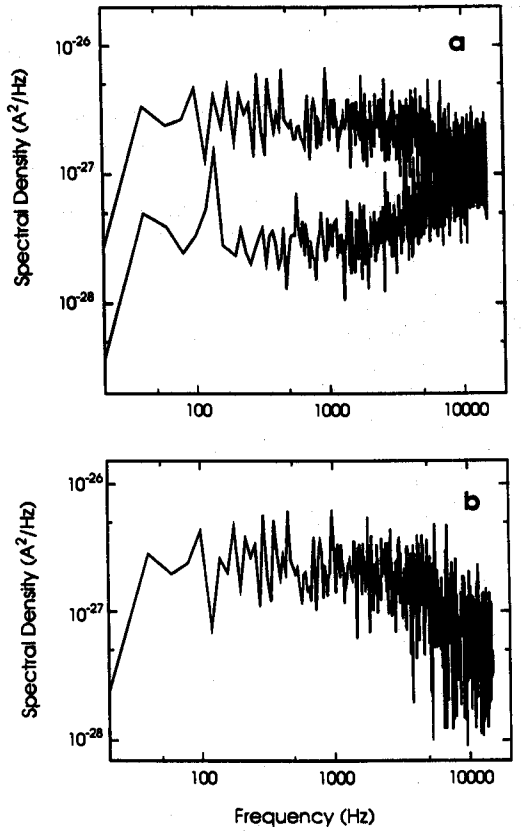

FIG. 3. (a) The spectral density of the open channel current noise (top trace) in comparison with the background (bottom trace) obtained from the same membrane immediately before the channel appeared at pH=5.9. (b) Difference noise spectrum showing a decline in the spectral density at $f>5$ kHz. Fast Fourier transforms were performed on 2048 point vectors, the corner frequency of the eight-pole Butterworth filter set to $\frac{3}{8}$ of the sampling frequency of 40 kHz.

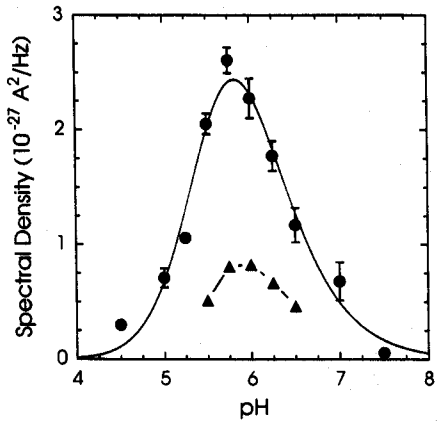

FIG. 4. Excess spectral density of current noise as a function of pH. The solutions bathing both sides of the bilayer contained $1M$ NaCl (filled circles), or $0.1M$ NaCl (filled triangles). The error bars represent the standard deviations of at least three experiments. Changing the chemical composition of the buffer, or using no buffer, had no significant effect on the noise (data not shown).

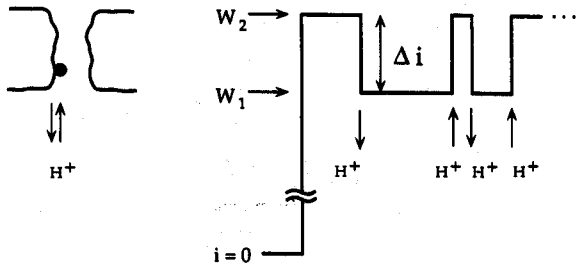

FIG. 5. Simplified model of reversible ionization reaction of groups in an α-toxin channel. Deprotonation of the channel causes a decrease in the current. One titratable site is shown a distance from the midpoint of the channel since the channel exhibits asymmetric I-V curves as a function of pH [12,13]. At low pH, state W_2 predominates because of the high proton concentration. At $p\mathrm{H}\sim p\mathrm{K}$, the channel switches, with equal probability, back and forth between states W_1 and W_2, causing maximum current noise.

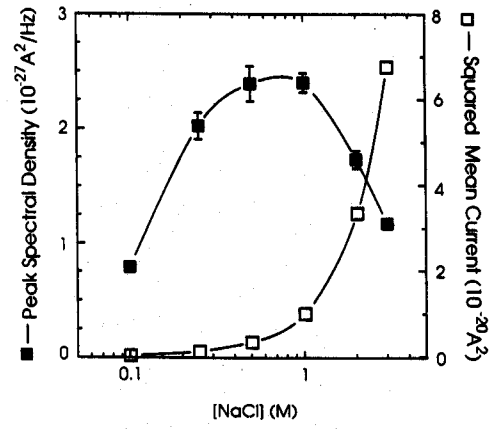

FIG. 6. Dependence of the peak excess noise spectral density (filled squares) and the squared mean current (open squares) through the channel on the concentration of NaCl. Saturation of the noise occurs at $0.5M$ NaCl, which corresponds to a Debye length approximately equal to the channel radius. Note, however, that the squared current only monotonically increases with increasing salt concentration.

current through the channel at the extremes in pH. In the low frequency limit, $(2\pi f\tau)^2 \ll 1$, the spectral density is

$$S_{i,H}(0) = 4(\Delta i)^2 \times 10^{p\mathrm{K}-p\mathrm{H}}/nk_D(1+10^{p\mathrm{K}-p\mathrm{H}})^3 . \quad (4)$$

Thus, fitting Eq. (4) to the measured pH-dependent spectral density (Fig. 4) allows one to estimate the effective dissociation rate constant nk_D and the pK.

The difference in current between the totally protonated and deprotonated states of the channel was measured directly from the single-channel currents at pH 4.5 and 7.5, and is $\Delta i = 2.0\times 10^{-11}$ A in $1M$ NaCl. The two-parameter least-squares fit for the $1M$ NaCl data with $nk_D = 1.0\times 10^5\ \mathrm{s}^{-1}$ is shown in Fig. 4 as the solid line. The agreement between this simple theory and experiment is excellent.

Using characteristic cutoff frequencies for measured spectra, we can determine the number of ionizable sites, n, which participate in this reaction. Indeed, according to Eqs. (2)-(4) we have

$$n = 4(\Delta i)^2 \tau 10^{p\mathrm{K}-p\mathrm{H}}/[S_{i,H}(0)(1+10^{p\mathrm{K}-p\mathrm{H}})^2] , \quad (5)$$

where τ is obtained from the inverse frequency at which the spectral density drops to one-half of its low-frequency value [see Eq. (3)]. By fitting the data in Fig. 3(b) to a Lorentzian function, we get $\tau = 3.1\times 10^{-5}$ s, and $n = 3.8$. Measurements performed at several different pH values yield an average value of $n = 4.2 \pm 0.7$.

We are now in a position to determine all of the main reaction parameters. Taking $n = 4$, we obtain $k_D = 2.5 \times 10^4\ \mathrm{s}^{-1}$. The inverse of this parameter, 4×10^{-5} s, equals the mean time a proton is bound to a single site. We can also deduce the association rate constant, $k_R = 8\times 10^9\ M^{-1}\mathrm{s}^{-1}$, since the equilibrium constant is defined by $K \equiv 10^{-p\mathrm{K}} = k_D/k_R$. This value of k_R is within an order of magnitude of the measured diffusion-limited rate constant of proton self-dissociation in water [16]. The change in the current through the open channel upon recharging of a single site is $\Delta i/n = 5\times 10^{-12}$ A, which contributes 5% of the total current through a single channel in $1M$ NaCl.

We made several simplifying assumptions in our model: The change in the channel current is proportional to the number of ionizable sites, the protonation of a given site is independent of the state of the other sites, and reaction rates are time independent. Experimental and simulation studies of proteins in many cases showed the existence of multiple states with continuous distribution of energy barriers or time-dependent energy barriers ([17,18], see also [19]). Still, the proposed first-order reaction model [Eqs. (1)-(4) and Fig. 5] describes adequately the results of our noise measurements. We can also exclude anomalies in electrolyte conductivity fluctuations related to high proton mobility [20] because their magnitude is far below the levels reported here.

It was reported earlier (for the dihydropyridine-sensitive Ca^{2+} channel from heart) that changing the pH influences the dynamics of that channel's transitions between different conductance states [21]. This phenomenon was attributed to the reversible binding of a proton to a single site on that channel which could modulate the conductance by either triggering a conformational change in the protein or by changing the local electrostatic potential in the pore itself. However, the authors could not distinguish between either of these possibilities.

One also might argue that the pH-dependent spectral densities in Fig. 4 are caused by structural fluctuations. In this case, at least for linear models of noise generation, the spectral density of current noise should be proportional to the square of the mean channel current [7]. However, this is not true (Fig. 6). This result permits us to con-

clude that noise generation in this channel is very close in nature to that in solid state microstructure electronic devices in which the capture and emission of carriers at a single defect site cause switching of the device's resistance due to changes in the potential barriers [8,9,22]. The data in Fig. 6 suggest that ionizable charged sites within the channel pore are electrostatically screened when the ionic concentration is increased from $0.5M$ to $3M$ NaCl. The characteristic screening Debye length, ~ 0.4 nm for a $0.5M$ 1:1 electrolyte in aqueous solution, is close to the estimated value for the channel radius [11,23].

It is tempting to speculate which sites in the protein pore are responsible for the pH-dependent current noise. Since the pK estimated in our noise measurements is 5.5, the groups that are the source of this excess noise are probably aspartic acids, glutamic acids, or histidines (but see [24]). Of course, there are examples of significant pK shifts of residues attached to proteins [25]. However, the values of the rate constants for association and dissociation that we deduced from our measurements are close to the values measured directly for carboxyl and imidazole groups in the bulk aqueous phase [26]. This suggests the titratable sites are not buried deeply within the protein core of the channel, and therefore would probably exhibit only a minor pK shift.

In conclusion, our experiments demonstrate the possibility of using noise analysis to study the kinetics of fast chemical reactions in a single microscopic (in fact, nanoscopic) "cuvette" in which only several molecules participate. To date, most studies of channel structure are performed by measuring the single-channel conductance and selectivity combined with genetic engineering [27]. We suggest that noise analysis of open-channel currents, coupled with site-directed mutagenesis, will prove useful as a novel probe of the functional structure of ion channels.

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