Statistical Mechanical Equilibrium Theory of Selective Ion Channels

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ABSTRACT A rigorous statistical mechanical formulation of the equilibrium properties of selective ion channels is developed, incorporating the influence of the membrane potential, multiple occupancy, and saturation effects. The theory provides a framework for discussing familiar quantities and concepts in the context of detailed microscopic models. Statistical mechanical expressions for the free energy profile along the channel axis, the cross-sectional area of the pore, and probability of occupancy are given and discussed. In particular, the influence of the membrane voltage, the significance of the electric distance, and traditional assumptions concerning the linearity of the membrane electric field along the channel axis are examined. Important findings are: 1) the equilibrium probabilities of occupancy of multiply occupied channels have the familiar algebraic form of saturation properties which is obtained from kinetic models with discrete states of denumerable ion occupancy (although this does not prove the existence of specific binding sites; 2) the total free energy profile of an ion along the channel axis can be separated into an intrinsic ion-pore free energy potential of mean force, independent of the transmembrane potential, and other contributions that arise from the interfacial polarization; 3) the transmembrane potential calculated numerically for a detailed atomic configuration of the gramicidin A channel embedded in a bilayer membrane with explicit lipid molecules is shown to be closely linear over a distance of 25 Å along the channel axis. Therefore, the present analysis provides some support for the constant membrane potential field approximation, a concept that has played a central role in the interpretation of flux data based on traditional models of ion permeation. It is hoped that this formulation will provide a sound physical basis for developing nonequilibrium theories of ion transport in selective biological channels.

INTRODUCTION

Recent progress in the determination of the three-dimensional structure of biological ion channels at atomic resolution gives a fresh impetus to efforts directed at understanding the fundamental principles governing ion permeation (Cowan et al., 1992; Doyle et al., 1998; Ketchem et al., 1997). Theoretical studies based on molecular dynamics (MD) simulations of atomic models can help us to better understand how ion channels function at the microscopic level (Brooks et al., 1988; Karplus and Petsko, 1990). The calculated classical trajectory, though an approximation to the real world, provides ultimate detailed information about the time course of the atomic motions, thus permitting a characterization of energetic and dynamic factors that are not easily accessible experimentally. Current methodologies have reached the point where one can generate trajectories of realistic atomic models of complex biological membrane systems (Tieleman and Berendsen, 1998; Tieleman et al., 1999; Woolf and Roux, 1994, 1996; Zhong et al., 1998). Molecular mechanical potential energy functions for detailed atomic models of proteins (MacKerell et al., 1998) and lipids (Schlenkrich et al., 1996), as well as fast and reliable simulation algorithms are available (Procacci et al., 1996). Nevertheless, despite the progress in computational methodologies theoretical investigations of ion channels are still confronted with difficult fundamental problems.

The first and main problem in simulating ion permeation is one of time scale. The translocation of a single ion across a channel takes on the order of a microsecond (Hille, 1992), which is extremely long compared to the typical length of calculated trajectories (Tieleman and Berendsen, 1998; Tieleman et al., 1999; Woolf and Roux, 1994, 1996; Zhong et al., 1998). A second problem is presented by the treatment of the membrane potential and its coupling to the ion movements. MD simulations of a realistic representation of the membrane potential, which arises from a very small imbalance of net charges of the mobile ions near the membrane-solution interface, would require a prohibitively large atomic system and are currently impractical (Roux, 1997). A last problem is the difficulty in identifying the relevant microscopic processes to simulate because it is likely that ion permeation in biological channels occurs via complex events involving several ions in a concerted fashion (Hille, 1992). The recent crystal structure of the KcsA K^+ channel, with three cations located in the pore, provides a striking example of the functional importance of multiple ion occupancy (Doyle et al., 1998). For these reasons, current MD calculations typically attempt to simulate some equilibrium state of ion channels rather than the complete nonequilibrium permeation process. To establish a connection with nonequilibrium transport properties, the results from the trajectories are often interpreted within the framework of simple phenomenological approaches, such as kinetic rate models (Heckmann, 1965a,b; Läuger, 1973; Parlin and Eyring, 1954; Zwolinski et al., 1949), or continuous electro-

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diffusion models (Chen et al., 1997; Goldman, 1943; Kurnikova et al., 1999; Levitt, 1986; McGill and Schumaker, 1996; Neumcke and Läuger, 1969). For example, the free energy profile of Na^+ in the gramicidin A (GA) channel calculated from MD simulations (Roux and Karplus, 1993) has been used as an input in a random walk diffusion model to calculate the current-voltage response (McGill and Schumaker, 1996). At the present time such simple models must be used for translating the results from MD simulations into electrophysiological observables. In fact, The situation is not specific to ion permeation. For example, statistical mechanical theories of transport are also established on a similar basis, with simple theories serving as a bridge between the microscopic and macroscopic levels (Helfand, 1960).

Statistical mechanical theories of nonequilibrium phenomena are generally constructed in terms of deviations from equilibrium states (Berne and Pecora, 1976). A rigorous formulation of the equilibrium state is thus an important first step in the establishment of any transport theory. Fundamental theories of ion permeation should be constructed according to similar guidelines. This would ensure, for example, that they are built on firm ground and return to a correct equilibrium state when external nonequilibrium conditions caused by the transmembrane potential or concentration gradients are removed. Current phenomenological theories of ion permeation, whether they are kinetic (Heckmann, 1965a,b; Läuger, 1973; Parlin and Eyring, 1954; Zwolinski et al., 1949) or electrodiffusion models (Chen et al., 1997; Kurnikova et al., 1999; Levitt, 1986; McGill and Schumaker, 1996), do not necessarily return to a satisfactory description of the equilibrium state of ion channels in the absence of ion fluxes. This is not surprising, because these theories attempt to describe very complex molecular systems with many simplifying assumptions (e.g., discrete states, mean-field potential, etc.).

A statistical mechanical formulation of the equilibrium state of ions in membrane channels can contribute to the clarification and improvement of current kinetic and electrodiffusion theories of ion transport. Furthermore, a characterization of equilibrium can be used for interpreting experimental ion flux data in the limit of zero current and is of interest in its own right. Analysis of the voltage-dependent and concentration-dependent equilibrium properties of an ion, in the absence of net fluxes, yields valuable information about the existence of favorable locations along the permeation pathway ("binding sites"), ion-ion interactions in the pore, probabilities of singly and multiply occupied states, and the coupling of ions to the transmembrane potential. In principle, MD calculations of detailed atomic models can be used to simulate ion channels at equilibrium. However, because a rigorous statistical mechanical formulation is lacking at the present time, it is not possible to make full use of the information provided by MD simulations. Clearly, a better characterization of the equilibrium state of ion channels at the microscopic level is needed.

The goal of this paper is to develop a rigorous statistical mechanical equilibrium theory of ions in membrane channels, including the influence of the membrane potential, ion-ion interactions, multiple occupancy, and saturation. Although the present paper is concerned only with equilibrium properties, it is hoped that the formulation will constitute a first step toward a comprehensive theory of nonequilibrium transport phenomena in ion channels. The present analysis provides a rigorous framework and helps to clarify the microscopic significance of familiar quantities and concepts in the context of detailed microscopic models. Statistical mechanical expressions are given for the free energy profile along the channel axis, the probability of singly and multiply occupied states, the equilibrium binding constant(s), and the cross-sectional area of a pore. In addition, the influence of the membrane voltage, the significance of the electric distance, and traditional assumptions concerning the linearity of the membrane electric field along the channel axis are examined in detail.

In the next section, the main theoretical developments are given. In the third section, the theoretical framework is discussed and illustrated in the context of the gramicidin A (GA) channel. The paper is concluded with a summary of the principal results in the fourth section.

THEORETICAL DEVELOPMENTS

Description of the microscopic system

An ion channel embedded in a lipid membrane in equilibrium with surrounding aqueous salt solutions is considered. The electrolyte solutions are not symmetrical, and there is a Nernst potential across the membrane. It is assumed that the channel is passively permeable to only one ionic species and remains in the open conducting state with no gating transitions; no other ions can pass through the channel or the membrane. Ideal selectivity of the channel to one ionic species is a necessary condition for a true equilibrium situation to exist in the presence of asymmetrical solutions. This is a direct extension of the concept of the perfectly semipermeable membrane, which is required for the existence of the equilibrium Nernst membrane potential (Hille, 1992; Roux, 1997). For example, the system could represent the GA channel bathed by KCl aqueous solutions of different concentrations, because this channel is virtually impermeable to anions (Hille, 1992). To proceed further with the statistical mechanical equilibrium theory, we assume that it is possible to identify a "pore" region from which all ions other than the permeating species are excluded, and a "bulk" region that contains the electrolytic solutions. The microscopic system is illustrated schematically in Fig. 1.

In thermodynamic equilibrium, a very small net charge accumulates on one side of the membrane, creating a membrane potential opposing the movement of the permeable ion (Roux, 1997). However, the bulk densities do not change significantly, and the solutions remain globally neutral, because any macroscopic charge imbalance in the bulk

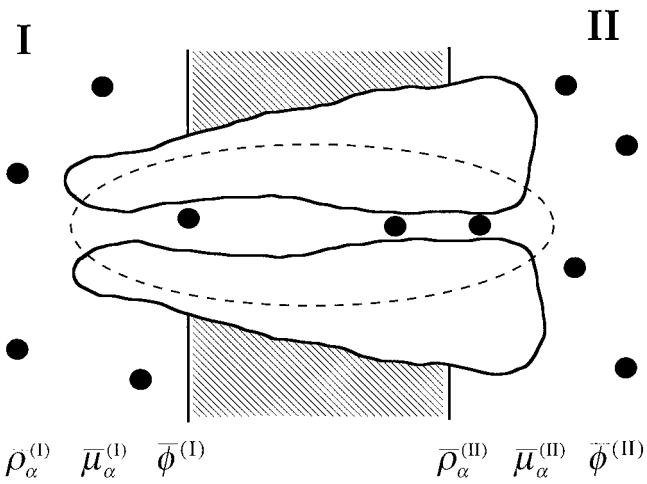

FIGURE 1 Schematic representation of the ion channel-membrane system with asymmetrical solutions on sides I and II. The "pore region," which corresponds to the ideally selective part of a channel, is highlighted with a dashed line. The "bulk region" corresponds to the remaining space in the system. The density of the permeable ions, the excess chemical potential, and the average electrostatic potential are, respectively, $\bar{\rho}^{(S)}$, $\bar{\mu}^{(S)}$, and $\bar{\phi}^{(S)}$ on the side S (I or II) of the membrane. For any instantaneous configuration, it is possible to know the number of ions occupying the pore region.

region would be energetically prohibitive. The density of the permeable ions on the side S (I or II) of the membrane is $\bar{\rho}^{(S)}$, and the chemical potential of the permeable ions on the side S (I or II) of the membrane is $\bar{\mu}^{(S)}$. For the permeable species,

$$\frac{\bar{\rho}^{(I)}}{\bar{\rho}^{(II)}} = \frac{e^{-\beta\bar{\mu}^{(I)}}}{e^{-\beta\bar{\mu}^{(II)}}}, \tag{1}$$

where $\beta = (k_B T)^{-1}$. In contrast, the chemical potential of the nonpermeating ions is not restricted by any conditions. The excess chemical potential of the permeable ion on side S is

$$\bar{\mu}^{(S)} = \Delta\bar{\mu}^{(S)} + q\bar{\phi}^{(S)}, \tag{2}$$

where q is the charge of the ion and $\Delta\bar{\mu}^{(S)}$ and $\bar{\phi}^{(S)}$ are the intrinsic excess chemical potential and the electrostatic potential in the bulk solution on side S, respectively. For example, the value of $\Delta\bar{\mu}^{(S)}$ corresponds roughly to the sum of Born and Debye-Hückel charging free energies (Roux, 1997). The former depends on the dielectric constant of the solvent, and the latter depends on the ionic strength of the salt solutions. The difference in the average electrostatic potential in the bulk solution is the Nernst membrane potential,

$$\begin{aligned}\bar{\phi}^{(II)} - \bar{\phi}^{(I)} &= \frac{k_B T}{q}\ln\left[\frac{\bar{\rho}^{(I)}}{\bar{\rho}^{(II)}}\right] + \frac{1}{q}\left[\Delta\bar{\mu}^{(I)} - \Delta\bar{\mu}^{(II)}\right] \\ &= \frac{k_B T}{q}\ln\left[\frac{\bar{\rho}^{(I)} e^{\beta\Delta\bar{\mu}^{(I)}}}{\bar{\rho}^{(II)} e^{\beta\Delta\bar{\mu}^{(II)}}}\right].\end{aligned} \tag{3}$$

By adjusting the composition of the salt solutions carefully, it is possible to balance the value of the intrinsic excess chemical potential on both sides and avoid differences in the activities. For the sake of simplicity, we assume that $V = \bar{\phi}^{(II)} - \bar{\phi}^{(I)}$ in the following.

The total potential energy of the entire system is $U(\mathbf{r}_1, \ldots, \mathbf{r}_N, \mathbf{X})$, where $(\mathbf{r}_1, \ldots, \mathbf{r}_N)$ are the coordinates of the N permeable ions and $\mathbf{X} \equiv \mathbf{X}_i, \mathbf{X}_w, \mathbf{X}_l, \mathbf{X}_c$ represent the remaining degrees of freedom in the system (i.e., $\mathbf{X}_i \equiv$ impermeable ions, $\mathbf{X}_w \equiv$ water molecules, $\mathbf{X}_l \equiv$ lipid molecules, and $\mathbf{X}_c \equiv$ channel). The permeating ions can translocate from one side to the other, whereas the nonpermeating ions cannot exchange from side I and side II. Because their number $N^{(S)}$ is fixed on each side S, their configurational integral is restricted to the side to which those ions are assigned. In contrast, the accessible configurational space of the permeating ions corresponds to the whole volume of the system.

Probability of occupancy and potential of mean force

By definition, the existence of the pore and bulk regions implies that any spatial integral over the whole volume V can be expressed as the sum of two integrals,

$$\int_V d\mathbf{r}\cdots \equiv \int_{pore} d\mathbf{r}\cdots + \int_{bulk} d\mathbf{r}\cdots. \tag{4}$$

From this definition, it follows that it is possible to determine the total number of permeating ions inside the pore for any instantaneous configuration of the pore system. It is given by the discrete function $n'(\mathbf{r}_1, \mathbf{r}_2, \ldots, \mathbf{r}_N)$, defined as

$$n'(\mathbf{r}_1, \mathbf{r}_2, \ldots, \mathbf{r}_N) = \int_{pore} d\mathbf{r} \sum_{i=1}^{N} \delta(\mathbf{r} - \mathbf{r}_i), \tag{5}$$

where $\mathbf{r}_i$ is the position of the ith ion, and the subscript of the integral sign implies that the integral is taken over the volume of the pore region. The probability, $\mathcal{P}_n$, of having exactly n ions inside the pore is calculated from the average,

$$\mathcal{P}_n = \langle \delta_{nn'} \rangle = \frac{\int d\mathbf{r}_1 \cdots \int d\mathbf{r}_N \int d\mathbf{X}\, \delta_{n,n'}\, e^{-\beta U}}{\int d\mathbf{r}_1 \cdots \int d\mathbf{r}_N \int d\mathbf{X}\, e^{-\beta U}}, \tag{6}$$

where $\delta_{nn'}$ is a Kronecker discrete delta function,

$$\delta_{nn'} = \begin{cases} 1 & \text{if } n = n'(\mathbf{r}_1, \mathbf{r}_2, \cdots \mathbf{r}_N) \\ 0 & \text{otherwise.} \end{cases} \tag{7}$$

By construction, the probabilities $\mathcal{P}_n$ are normalized, i.e., $\Sigma_n \mathcal{P}_n = 1$, via the completeness of the Kronecker delta. It follows that the average of any observable A may be expressed as a weighted sum over the occupancy states of the channel,

$$\langle A \rangle = \sum_n \mathcal{P}_n \langle A \rangle_{(n)}, \tag{8}$$

where $\langle A\rangle_{(n)}$ is the average of A when exactly n ions are in the pore region. For example, A could be a spectroscopic observable such as a NMR chemical shift (Hinton et al., 1988; Jing et al., 1995; Tian et al., 1996; Woolf and Roux, 1997).

To determine the probabilities of occupancy, it is useful to consider the binding factor $\mathcal{B}_n$ corresponding to the ratio $\mathcal{P}_n/\mathcal{P}_0$. For $n = 1$, this is

$$\mathcal{B}_1 = \frac{\int d\mathbf{r}_1 \ldots \int d\mathbf{r}_N \int d\mathbf{X}\, \delta_{1,n'} e^{-\beta U}}{\int d\mathbf{r}_1 \ldots \int d\mathbf{r}_N \int d\mathbf{X}\, \delta_{0,n'} e^{-\beta U}}, \tag{9}$$

(the expression for $\mathcal{P}_n$ in Eq. 6 has been used). Because the factor $\delta_{1,n'}$ in the integrand is zero unless one of the N ions is located inside the pore, the expression may be rewritten as

$$\mathcal{B}_1 = N \frac{\int_{pore} d\mathbf{r}_1 \int_{bulk} d\mathbf{r}_2 \cdots \int_{bulk} d\mathbf{r}_N \int d\mathbf{X}\, e^{-\beta U}}{\int_{bulk} d\mathbf{r}_1 \int_{bulk} d\mathbf{r}_2 \cdots \int_{bulk} d\mathbf{r}_N \int d\mathbf{X}\, e^{-\beta U}}, \tag{10}$$

where the ion number 1 was chosen arbitrarily to occupy the pore. The factor of N is included to account for the multiple ways to obtain equivalent configurations. Similarly, the n-ion binding factor $\mathcal{B}_n$ is

$$\mathcal{B}_n = \frac{N!}{n!(N-n)!} \times \frac{\int_{pore} d\mathbf{r}_1 \cdots \int_{pore} d\mathbf{r}_n \int_{bulk} d\mathbf{r}_{n+1} \cdots \int_{bulk} d\mathbf{r}_N \int d\mathbf{X}\, e^{-\beta U}}{\int_{bulk} d\mathbf{r}_1 \ldots \int_{bulk} d\mathbf{r}_N \int d\mathbf{X}\, e^{-\beta U}}, \tag{11}$$

because there are $N!/(n!(N-n)!)$ equivalent configurations with identical ions. In the thermodynamic limit, $N \to \infty$, and the prefactor $(N-n)!/n! \approx N^n/n!$.

The one-ion binding factor may be expressed as

$$\mathcal{B}_1 = N \frac{\int_{pore} d\mathbf{r}_1\, e^{-\beta \mathcal{W}(\mathbf{r}_1)}}{\int\int_{\mathbf{bulk}} \mathbf{dr}_1\, e^{-\beta \mathcal{W}(\mathbf{r}_1)}}, \tag{12}$$

where $\mathcal{W}(\mathbf{r}_1)$ is the potential of mean force (PMF) with one ion inside the pore. The PMF corresponds to the reversible thermodynamic work needed to adiabatically move an ion into the pore region. Its first derivative is equal to minus the average (mean) force exerted on a permeating ion by the channel, the water, the other counterions, and the membrane, i.e., $\langle \mathbf{F}\rangle = -\nabla \mathcal{W}$. In that sense, the PMF is not equal to an average potential energy but to a free energy. The concept of the PMF was originally introduced by Kirkwood (1935) to describe the structure of liquids. Such a reversible work function currently plays a key role in modern statistical mechanical theories of equilibrium and nonequilibrium processes in molecular systems (Chandler, 1978) in general, and in ion transport (Roux and Karplus, 1991a,b) in particular. Here we are only concerned with the relation of the PMF to the equilibrium properties.

According to Eq. 12, the PMF is determined relative to an arbitrary offset constant. To have a simple relationship with the excess chemical potential of the ion in the bulk solution, we choose to define the PMF relative to a system with one noninteracting ion,

$$e^{-\beta \mathcal{W}(\mathbf{r}_1)} = \frac{\int_{bulk} d\mathbf{r}_2 \cdots \int_{bulk} d\mathbf{r}_N \int d\mathbf{X}\, e^{-\beta U}}{\int_{bulk} d\mathbf{r}_2 \cdots \int_{bulk} d\mathbf{r}_N \int d\mathbf{X}\, e^{-\beta U_1^*}}, \tag{13}$$

where the notation U_1^* means that all interactions involving ion 1 with the rest of the system have been switched off. This procedure is formally similar to that used in alchemical free energy molecular dynamics techniques (e.g., see Kollman, 1993, and references therein). Note that, by construction, $\mathcal{W}(\mathbf{r}_1) \to \bar{\mu}^{(S)}$ as $\mathbf{r}_1$ goes to side S at a large distance from the pore. The volume integral over the bulk region is

$$\int_{bulk} d\mathbf{r}_1\, e^{-\beta \mathcal{W}(\mathbf{r}_1)} = V^{(I)} e^{-\beta \bar{\mu}^{(I)}} + V^{(II)} e^{-\beta \bar{\mu}^{(II)}} \tag{14}$$

$$= \frac{N}{\bar{\rho}^{(I)}} e^{-\beta \bar{\mu}^{(I)}}$$

$$= \frac{N}{\bar{\rho}^{(II)}} e^{-\beta \bar{\mu}^{(II)}}, \tag{15}$$

because $N = V^{(I)}\bar{\rho}^{(I)} + V^{(II)}\bar{\rho}^{(II)}$ and $e^{-\beta\bar{\mu}^{(II)}} = (\bar{\rho}^{(II)}/\bar{\rho}^{(I)})e^{-\beta\bar{\mu}^{(I)}}$, according to Eq. 1. Because the density and the excess chemical potential at equilibrium are related via Eq. 1, it is possible to express the ratio $\mathcal{B}_1$ in terms of $\bar{\rho}^{(I)}$ or, equivalently, $\bar{\rho}^{(II)}$:

$$\mathcal{B}_1 = \bar{\rho}^{(I)} \int_{pore} d\mathbf{r}_1\, e^{-\beta[\mathcal{W}(\mathbf{r}_1) - \bar{\mu}^{(I)}]}$$

$$= \bar{\rho}^{(II)} \int_{pore} d\mathbf{r}_1\, e^{-\beta[\mathcal{W}(\mathbf{r}_1) - \bar{\mu}^{(II)}]}. \tag{16}$$

Similarly, the n-ion binding factor is

$$\mathcal{B}_n = (\bar{\rho}^{(I)})^n \frac{1}{n!} \int_{pore} d\mathbf{r}_1 \cdots \int_{pore} d\mathbf{r}_n\, e^{-\beta[\mathcal{W}(\mathbf{r}_1, \cdots \mathbf{r}_n) - n\bar{\mu}^{(I)}]}, \tag{17}$$

where the n-ion PMF has been defined relative to n noninteracting ions:

$$e^{-\beta \mathcal{W}(\mathbf{r}_1, \cdots \mathbf{r}_n)} = \frac{\int_{bulk} d\mathbf{r}_{n+1} \cdots \int_{bulk} d\mathbf{r}_N \int d\mathbf{X}\, e^{-\beta U}}{\int_{bulk} d\mathbf{r}_{n+1} \cdots \int_{bulk} d\mathbf{r}_N \int d\mathbf{X}\, e^{-\beta U^*_{1,\cdots,n}}}, \tag{18}$$

where the notation indicates that all interactions involving ion 1, . . . , n have been switched off in the energy $U^*_{1,\ldots,n}$.

Once the binding factors $\mathcal{B}_n$ have been determined, the probability of any state of occupancy can be obtained using $\mathcal{B}_0 = 1$ with the normalization condition

$$\langle \delta_{n,n} \rangle = \langle \delta_{0,n'} \rangle \mathcal{B}_n, \tag{19}$$

yielding

$$\mathcal{P}_n = \frac{\mathcal{B}_n}{1 + \mathcal{B}_1 + \mathcal{B}_2 + \mathcal{B}_3 + \cdots}. \quad (20)$$

In particular, the probability that the pore is unoccupied is

$$\mathcal{P}_0 = \frac{1}{1 + \mathcal{B}_1 + \mathcal{B}_2 + \mathcal{B}_3 + \cdots}. \quad (21)$$

The denominator in Eqs. 20 and 21 may be expressed in the form of an effective Grand Canonical Partition function of an open finite system in contact with a bath of particles,

$$\Xi = \sum_{n=0}^{\infty} e^{n\beta\bar{\mu}^{(I)}} (\bar{\rho}^{(I)})^n \frac{1}{n!} \int_{pore} d\mathbf{r}_1 \cdots \int_{pore} d\mathbf{r}_n \, e^{-\beta \mathcal{W}(\mathbf{r}_1, \cdots \mathbf{r}_n)}. \quad (22)$$

Equation 22 provides a compact and useful notation for handling the multiion configurational distribution functions in the pore system.

For any realistic channel, all of the probabilities of occupancy $\mathcal{P}_n$ must necessarily be zero if n is larger than some value N_{max}, the maximum number of ions that can occupy the pore simultaneously. One important special case, the so-called one-ion pore theory (Läuger, 1973; Levitt, 1986; McGill and Schumaker, 1996), occurs if it is assumed that the pore cannot be occupied by more than one ion. It follows that all of the binding factors $\mathcal{B}_2 = \mathcal{B}_3 \ldots = 0$, and the probability of finding one ion inside the pore is simply

$$\mathcal{P}_1 = \frac{\mathcal{B}_1}{1 + \mathcal{B}_1}. \quad (23)$$

This equation can be compared with the familiar expression for first-order saturation for substrate binding,

$$\mathcal{P}_1 = \frac{\bar{\rho}^{(I)} K_1}{1 + \bar{\rho}^{(I)} K_1} \quad (24)$$

(expressed in terms of side I), where K_1 is the one-ion binding constant,

$$K_1 = \int_{pore} d\mathbf{r}_1 \, e^{-\beta[\mathcal{W}(\mathbf{r}_1) - \bar{\mu}^{(I)}]} \quad (25)$$

The n-ion binding constants K_n can be defined for multiply occupied channels in a similar fashion.

Influence of the membrane potential

So far, our treatment describes the most general situation with asymmetrical solutions and a Nernst membrane potential. Symmetrical solutions with no membrane potential correspond to a particularly important special case. It may be anticipated that the equilibrium properties in the general situation can be expressed in terms of a dominant contribution corresponding to the symmetrical solutions with no membrane potential, plus other contributions associated with the transmembrane potential.

For this purpose, we separate the system into a pore subsystem and the rest. One purpose of this separation is to enable us to use a continuum electrostatics approximation to describe the transmembrane potential (see below). For the sake of simplicity, we focus on the one-ion PMF, although the treatment can be easily generalized to the n-ion PMFs described in the previous section. The subsystem is constituted by the ion, the channel, and the m nearest solvent molecules in the pore region. Let $\mathbf{X}_{w/p}$ represent the degrees of freedom of the m nearest water molecules located inside the pore region, and let $\mathbf{X}_{w/b}$ represent those of the remaining water molecules in the bulk region. The degrees of freedom of the pore subsystem are represented by $\mathbf{X}_p \equiv \mathbf{r}_1$, $\mathbf{X}_c$, $\mathbf{X}_{w/p}$, those of the rest by $\mathbf{X}_r \equiv \mathbf{r}_2, \ldots, \mathbf{r}_N, \mathbf{X}_1, \mathbf{X}_{w/b}$. The permeating ion is particle 1 according to the notation. The potential energy U may be written as the sum of three contributions, $U = U_p(\mathbf{X}_p) + U_{pr}(\mathbf{W}_p, \mathbf{X}_r) + U_r(\mathbf{X}_r)$.

The statistical properties of such a finite subsystem representation of an infinite thermodynamic system have been formulated previously (Beglov and Roux, 1994). For a fixed configuration, the free energy of the ion and the subsystem in the membrane potential is

$$e^{-\beta \mathcal{F}(\mathbf{X}_p)} = \frac{\int d\mathbf{X}_r \, H_p(\mathbf{X}_r) e^{-\beta[U_p(\mathbf{X}_p) + U_{pr}(\mathbf{X}_p, \mathbf{X}_r) + U_r(\mathbf{X}_r)]}}{\int d\mathbf{X}_r \, e^{-\beta U_r(\mathbf{X}_r)}}, \quad (26)$$

where $H_p(\mathbf{X}_r)$ is a Heaviside step function that prevents the permeating ions (2, . . ., N) as well as the impermeable ions from penetrating the pore region p. That is, $H_p(\mathbf{X}_r) = 0$ if any of those particles is located in the pore region, in accord with Eq. 4. In addition, the function $H_p(\mathbf{X}_r)$ restricts the configuration of the bulk solvent molecules so that they remain farther than the m nearest molecules (see Beglov and Roux, 1994, for a discussion of restricted configurational integrals). In the general case, the free energy function $\mathcal{F}(\mathbf{X}_p)$ depends on the density of ions on side I and side II, i.e., $\mathcal{F}(\mathbf{X}_p) = \mathcal{F}(\mathbf{X}_p; \bar{\rho}^{(I)}, \bar{\rho}^{(II)})$. Under symmetrical conditions with no membrane potential, the free energy is $\mathcal{F}_{sym}(\mathbf{X}_p) \equiv \mathcal{F}(\mathbf{X}_p; \bar{\rho}^{(I)} = \bar{\rho}^{(II)} = \bar{\rho})$.

The transmembrane potential results from long-range electrostatic interactions due to a very small imbalance of net charges, involving the mobile ions in the bulk solutions on each sides of the membrane. To describe its influence on the PMF, it is necessary to use some approximation. A possible approach is to use a continuum electrostatic treatment based on the Poisson-Boltzmann equation modified to account for an equilibrium Nernst membrane potential (Roux, 1997),

$$\nabla \cdot [\epsilon(\mathbf{r}) \nabla \phi(\mathbf{r}) - \bar{\kappa}^2(\mathbf{r})[\phi(\mathbf{r}) - V\Theta(\mathbf{r})]] = -4\pi\lambda\rho_p(\mathbf{r}), \quad (27)$$

where $\Theta(\mathbf{r})$ is a step function equal to one on side II and zero otherwise, $\bar{\kappa}^2(\mathbf{r})$ is the space-dependent screening factor, $\rho_p(\mathbf{r})$ is the charge density of the pore subsystem, and λ is a coupling parameter. The step function ensures that

mobile ions are in equilibrium with the bath with which they are in contact (on the right side the reference voltage is zero; on the left side the reference voltage is V). A closed-form expression for the free energy $\mathcal{F}(\mathbf{X}_p)$ of a macromolecular subsystem in the membrane field has been derived based on Eq. 27:

$$\mathcal{F}(\mathbf{X}_p) = U_p(\mathbf{X}_p) + \mathcal{F}_{cavity} + \frac{1}{2}CV^2 + \left[\sum_p q_p\phi_{mp}(\mathbf{r}_p)\right]V + \frac{1}{2}\left[\sum_p q_p\phi_{rf}(\mathbf{r}_p)\right], \quad (28)$$

where the first term is the microscopic energy of the pore subsystem, the second term is the free energy associated with the creation of a cavity to insert the neutral subsystem in the membrane, the third term is the free energy needed to charge up the capacitance C of the neutral subsystem, the fourth term represents the interaction of the protein charges with the membrane potential (calculated in the absence of the charge of the subsystem with $\lambda = 0$ and $V = 1$),

$$\nabla\cdot[\epsilon(\mathbf{r})\nabla\phi_{mp}(\mathbf{r})] - \bar{\kappa}^2(\mathbf{r})[\phi_{mp}(\mathbf{r}) - \Theta(\mathbf{r})] = 0, \quad (29)$$

and the fifth and last term represents the reaction field due to the solvent. The reaction field is computed as the difference between the potential ϕ in the complete environment and vacuum (Honig and Nicholls, 1995; Nina et al., 1997), i.e., $\phi_{rf} = \phi(\text{env}) - \phi(\text{vac})$, where ϕ is the solution to the standard PB equation with no membrane potential ($V = 0$ and $\lambda = 1$):

$$\nabla\cdot[\epsilon(\mathbf{r})\nabla\phi(\mathbf{r})] - \bar{\kappa}^2(\mathbf{r})\phi(\mathbf{r}) = -4\pi\rho_p(\mathbf{r}). \quad (30)$$

The capacitive contribution is negligibly small and can be ignored in the present case (Roux, 1997). The cavity term is independent of the membrane potential and is roughly proportional to the solvent-exposed surface area (Ben-Tal et al., 1996). It should be emphasized that $\mathcal{F}(\mathbf{X}_p)$ in Eq. 28 depends on the microscopic configuration $\mathbf{X}_p$ of the whole content of the pore (ion, channel, and water) defining the pore region. Equations 27, 29, and 30 have forms similar to that of the traditional linearized Poisson-Boltzmann equation (Honig and Nicholls, 1995). The space-dependent dielectric function $\epsilon(\mathbf{r})$ and Debye screening factor $\kappa(\mathbf{r})$ can be constructed following a standard prescription with the solvent-excluded molecular surface (Nina et al., 1997). In addition, the Heaviside step function H_p forces the value of $\bar{\kappa}(\mathbf{r})$ to be zero and the value of $\epsilon(\mathbf{r})$ to be one in the pore region, in accord with Eq. 26. One may note that the influence of explicit charges in the pore region and the transmembrane potential are superimposable on the free energy Eq. 28 because the modified PB Eq. 27 has been linearized. This has been pointed out previously (Jordan et al., 1989).

Following Eqs. 13 and 26, the one-ion PMF may be expressed as

$$e^{-\beta\mathcal{W}(\mathbf{r}_1)} = \frac{\int d\mathbf{X}_p'\,\delta(\mathbf{r}_1-\mathbf{r}_1')e^{-\beta\mathcal{F}(\mathbf{X}_p')}}{\int d\mathbf{X}_p'\,\delta(\mathbf{r}_1-\mathbf{r}_1')e^{-\beta\mathcal{F}^*(\mathbf{X}_p')}}, \quad (31)$$

where the notation $\mathcal{F}^*$ means that all interactions involving the ion with all atoms in the system (bulk and pore) have been switched off. In the general case, the PMF is $\mathcal{W}(\mathbf{r}_1)$ and depends on all conditions on the system. In the special case of symmetrical electrolyte solutions and no membrane potential, the PMF is $\mathcal{W}^{(0)}(\mathbf{r}_1)$, which we call the "intrinsic ion-pore PMF." We seek an expression for the difference $\mathcal{W}(\mathbf{r}_1) - \mathcal{W}^{(0)}(\mathbf{r}_1)$,

$$e^{-\beta[\mathcal{W}(\mathbf{r}_1)-\mathcal{W}^{(0)}(\mathbf{r}_1)]} = \frac{\int d\mathbf{X}_p'\,\delta(\mathbf{r}_1-\mathbf{r}_1')e^{-\beta\mathcal{F}(\mathbf{X}_p')}}{\int d\mathbf{X}_p'\,\delta(\mathbf{r}_1-\mathbf{r}_1')e^{-\beta\mathcal{F}^*(\mathbf{X}_p')}} \times \frac{\int d\mathbf{X}_p'\,\delta(\mathbf{r}_1-\mathbf{r}_1')e^{-\beta\mathcal{F}^*_{sym}(\mathbf{X}_p')}}{\int d\mathbf{X}_p'\,\delta(\mathbf{r}_1-\mathbf{r}_1')e^{-\beta\mathcal{F}_{sym}(\mathbf{X}_p')}} \quad (32)$$

$$= \frac{\int d\mathbf{X}_p'\,\delta(\mathbf{r}_1-\mathbf{r}_1')e^{-\beta\mathcal{F}(\mathbf{X}_p')}}{\int d\mathbf{X}_p'\,\delta(\mathbf{r}_1-\mathbf{r}_1')e^{-\beta\mathcal{F}_{sym}(\mathbf{X}_p')}} \times \frac{\int d\mathbf{X}_p'\,\delta(\mathbf{r}_1-\mathbf{r}_1')e^{-\beta\mathcal{F}^*_{sym}(\mathbf{X}_p')}}{\int d\mathbf{X}_p'\,\delta(\mathbf{r}_1-\mathbf{r}_1')e^{-\beta\mathcal{F}^*(\mathbf{X}_p')}}$$

$$= \langle e^{-\beta\Delta\mathcal{F}}\rangle_{\mathcal{F}_{sym};\mathbf{r}_1)} \times \langle e^{-\beta\Delta\mathcal{F}^*}\rangle^{-1}_{(\mathcal{F}^*_{sym})},$$

where $\Delta\mathcal{F} = \mathcal{F} - \mathcal{F}_{sym}$ and $\Delta\mathcal{F}^* = \mathcal{F}^* - \mathcal{F}^*_{sym}$ are the excess perturbation free energy contributions caused by asymmetrical conditions relative to symmetrical systems. The bracket with subscript $(\mathcal{F}_{sym};\mathbf{r}_1)$ represents an average,

$$\langle\cdots\rangle_{(\mathcal{F}_{sym};\mathbf{r}_1)} \equiv \frac{\int d\mathbf{X}_p'\cdots\delta(\mathbf{r}_1-\mathbf{r}_1')e^{-\beta\mathcal{F}_{sym}(\mathbf{X}_p')}}{\int d\mathbf{X}_p'\,\delta(\mathbf{r}_1-\mathbf{r}_1'e^{-\beta\mathcal{F}_{sym}(\mathbf{X}_p')}} \quad (33)$$

with the ion fixed at $\mathbf{r}_1$; the configurations are Boltzmann-weighted by the free energy $\mathcal{F}_{sym}$ of a symmetrical system. A similar expression holds for the configurational averages performed with the free energy $\mathcal{F}^*_{sym}$. Note that in the latter case the subscript $\mathbf{r}_1$ can be dropped because averages are equivalent to those that would be calculated in the absence of ion inside the pore, because its interactions have been switched off.

We must now evaluate the excess perturbation free energies, $\Delta\mathcal{F}$ and $\Delta\mathcal{F}^*$. In fact, their form is remarkably simple. The cavity formation $\mathcal{F}_{cavity}$ does not contribute because it is independent of the membrane potential. Furthermore, even though the reaction field free energy arises from long-range electrostatic interactions between the charges in the pore subsystem and the environment, it does not contribute to the excess perturbations if the ionic strength of the solution is kept unchanged on both sides of the membrane as the transmembrane potential is applied. It follows that

$$\Delta\mathcal{F}(\mathbf{X}_p) = V\left[\sum_p q_p\phi_{mp}(\mathbf{r}_p)\right] = V\left[q_1\phi_{mp}(\mathbf{r}_1) + \sum_{p>1} q_p\phi_{mp}(\mathbf{r}_p)\right], \quad (34)$$

where particle 1 is the ion (the sum with $p > 1$ runs over all particles other than the ion). Similarly,

$$\Delta\mathcal{F}^*(\mathbf{X}_p) = V\left[\sum_{p>1} q_p\phi_{mp}(\mathbf{r}_p)\right], \quad (35)$$

because the interactions of the ion with the surrounding are switched off. The membrane potential $\phi_{mp}(\mathbf{r})$ represents the influence of the polarization of the counterions in the solute at the membrane-bulk interface. It is calculated with Eq. 29, i.e., in the absence of the charge of the subsystem.

It is possible to treat the couplings $\Delta\mathcal{F}$ and $\Delta\mathcal{F}^*$ perturbatively and express the complete PMF as a series in increasing powers of the membrane potential V. To this end, we develop the exact expression Eq. 32 in terms of a cumulant expansion (Balescu, 1975),

$$\langle e^{-\beta\Delta\mathcal{F}}\rangle = e^{-\beta\langle\Delta\mathcal{F}\rangle+\beta^2[\langle\Delta\mathcal{F}^2\rangle-\langle\Delta\mathcal{F}\rangle^2]/2+\cdots} \quad (36)$$

(the subscripts on the bracket have been omitted for the sake of clarity). Such a cumulant expansion does not require that the perturbation be small, but that the structure of the coupling with the reference system be simple. A cumulant expansion is usually a good approximation if high-order terms are small (asymmetry of the distribution, etc.). For example, truncating the cumulant expansion to second order as in Eq. 36 corresponds to the quasiharmonic approximation for the fluctuations of proteins (Ichiye and Karplus, 1987), which would be rigorously exact if the fluctuations of the pore subsystem were Gaussian. From Eq. 32 the PMF is to lowest order in the perturbation,

$$\mathcal{W}(\mathbf{r}_1) = \mathcal{W}^{(0)}(\mathbf{r}_1) + \mathcal{W}^{(1)}(\mathbf{r}_1) + \mathcal{W}^{(2)}(\mathbf{r}_1) + \cdots, \quad (37)$$

where the first-order term is

$$\begin{aligned}\mathcal{W}^{(1)}(\mathbf{r}_1) &= \langle\Delta\mathcal{F}\rangle_{(\mathcal{F}_{sym};\mathbf{r}_1)} - \langle\Delta\mathcal{F}\rangle_{(\mathcal{F}^*_{sym})} \\ &= V\left[q_1\phi_{mp}(\mathbf{r}_1) + \left\langle\sum_{p>1} q_p\phi_{mp}(\mathbf{r}_p)\right\rangle_{(\mathcal{F}_{sym};\mathbf{r}_1)} \right. \\ &\qquad \left. - \left\langle\sum_{p>1} q_p\phi_{mp}(\mathbf{r}_p)\right\rangle_{(\mathcal{F}^*_{sym})}\right], \quad (38)\end{aligned}$$

(the explicit expressions for $\Delta\mathcal{F}$ and $\Delta\mathcal{F}^*$ have been used). Thus, to linear order in V, the influence of the membrane potential arises from the interaction of all of the charges of the pore subsystem (i.e., the ion, the channel, and its water content) with the field ϕ_{mp}. The second-order term (quadratic in V) is due to the influence of fluctuations,

$$\begin{aligned}\mathcal{W}^{(2)}(\mathbf{r}_1) = -\frac{1}{2k_BT}\left[\langle\Delta\mathcal{F}^2\rangle_{(\mathcal{F}_{sym};\mathbf{r}_1)} - \langle\Delta\mathcal{F}\rangle^2_{(\mathcal{F}_{sym};\mathbf{r}_1)} - \langle\Delta\mathcal{F}^2\rangle_{(\mathcal{F}^*_{sym})}\right. \\ \left. + \langle\Delta\mathcal{F}\rangle^2_{(\mathcal{F}^*_{sym})}\right]. \quad (39)\end{aligned}$$

Because the ion is fixed at $\mathbf{r}_1$, the direct term $q_1\phi_{mp}(\mathbf{r}_1)$ does not contribute to the fluctuations in Eq. 39 (although the presence of the ion at $\mathbf{r}_1$ has a direct influence on the fluctuations of the remaining components inside the pore. The second-order contribution is quadratic in the membrane potential V, which is analogous to a capacitive energy (Sigworth, 1993). It can be shown that it is related to the linear response of the charge distribution in the pore region due to the influence of the transmembrane field (Balescu, 1975). Although the analysis was carried out for the case of one ion inside the pore, the influence of the membrane potential on a multiply occupied state with n ions can be derived using a similar route (see the discussion in the next section).

Reduction to a one-dimensional system

With current computers, calculation of the function $\mathcal{W}(\mathbf{r})$ at a large number of positions $\mathbf{r} \equiv (x, y, z)$ of the permeating ion in three-dimensional space (the subscript 1 is omitted for simplicity) is nearly intractable. Ion permeation is generally discussed in terms of $w(x)$, the free energy profile of ions along the channel axis. The function $w(x)$ is an important input in kinetic rate models (Läuger, 1973) and in the one-dimensional Nernst-Planck equation (Levitt, 1986; McGill and Schumaker, 1996). From a dynamical point of view, the reduction in dimensionality is based on the assumption that that motions perpendicular to x reach equilibrium rapidly and that x is the only slow variable in the system (i.e., it plays the role of a reaction coordinate). However, discussions of the equilibrium free energy profile along the channel axis require no such assumptions about a separation of time scale. From Boltzmann statistics, the free energy profile, $w_{prj}(x)$, expressed as a projection of the complete PMF $\mathcal{W}(\mathbf{r})$ coordinate x, is

$$e^{-\beta w_{prj}(x)} = e^{-\beta[\mathcal{W}(x_c,y_c,z_c)-\bar{\mu}^{(I)}]}\frac{\int dy\, dz\, e^{-\beta\mathcal{W}(x,y,z)}}{\int dy\, de\, e^{-\beta\mathcal{W}(x_c,y,z)}}. \quad (40)$$

The free energy profile in Eq. 40 is normalized such that the value of $w_{prj}(x)$ at the position x_c is equal to the relative free energy $[\mathcal{W}(\mathbf{r}) - \bar{\mu}^{(I)}]$ of an ion at the point $\mathbf{r}_c$, chosen arbitrarily at some position along the axis of the channel. A definition of the cross section of the pore, S, follows from the choice of the reference point $\mathbf{r}_c$:

$$S = \int dy\, dz\, e^{-\beta[\mathcal{W}(x_c,y,z)-\mathcal{W}(x_c,y_c,z_c)]}. \quad (41)$$

This definition is convenient for establishing a link with well-known expressions for the one-ion equilibrium binding constant in terms of the free energy profile and the cross-sectional area of the channel (Levitt, 1986),

$$K_1 = S\int_{pore} dx\, e^{-\beta w_{prj}(x)} \quad (42)$$

which is equivalent to Eq. 25. The value of the cross-sectional area involved in the definition of the binding

constant differs from the cross-sectional area of the pore estimated on the basis of the exclusion radius of the channel atoms (Smart et al., 1993). In particular, its value depends on both the channel structure and the radius of the ion. According to this analysis, a single value is defined for the cross-sectional area of the pore. Alternatively, one could define a cross-sectional area $S(x)$ that varies along the x axis,

$$S(x) = \int \mathrm{d}y\, \mathrm{d}z\, e^{-\beta[\mathcal{W}(x,y,z)-\mathcal{W}[x,y_c,z_c)]}. \qquad (43)$$

From this choice, the binding constant is

$$K_1 = \int_{\text{pore}} \mathrm{d}x\, S(x) e^{-\beta w_{\text{axi}}(x)}, \qquad (44)$$

which implies that the appropriate definition of the free energy profile following from this construction would have to be the value of the PMF along the channel axis, $w_{\text{axi}}(x) = [\mathcal{W}(x, y_c, z_c) - \bar{\mu}]$, to recover the correct one-ion binding constant K_1. Note that $w_{\text{axi}}(x)$ differs from $w_{\text{prj}}(x)$ based on Eq. 40.

Although there is definitely a certain element of arbitrariness in the definition of a suitable free energy profile and cross-sectional area along the channel axis, it is important that Eqs. 41 and 43 remain consistent with Eq. 25. In kinetic rate models the cross-sectional area is related to the concept of capture radius (Läuger, 1973). A projection of the three-dimensional configurational space of an ion onto a single variable x is meaningful only in the pore region, where the lateral displacements of the ion (along y and z) are bounded. The extension of free energy profiles to the bulk region has no significance and actually diverges because the ion has infinitely more lateral freedom in the bulk region than inside the pore. Nevertheless, one-dimensional free energy profiles can be very useful concepts.

DISCUSSION

In the following, the main results concerning the probabilities of multiply occupied states, the definition of the free energy PMF, the effect of the transmembrane potential and its linearity, and the reduction to a one-dimensional system are reviewed and discussed. Where possible, the results are illustrated in the context of the GA channel.

Probabilities of occupancy

The present analysis, based on equilibrium statistical mechanical considerations, demonstrates that the probability of multiply occupied states, with explicit numbers of ions, can be expressed as

$$\mathcal{P}_n = \frac{K_n (\bar{\rho}^{(I)})^n}{1 + K_1 (\bar{\rho}^{(I)}) + K_2 (\bar{\rho}^{(I)})^2 + K_3 (\bar{\rho}^{(I)})^3 + \cdots}. \qquad (45)$$

The form of $\mathcal{P}_n$ is very similar to that of the familiar saturation expressions obtained from kinetic models. This surprising result may be understood simply. Kinetic models are constructed on the basis of two ingredients: first, it is assumed that the total configurational space of the whole system is constituted of a complete collection of distinct subspaces (the states); second, it is assumed that the system possesses no dynamical memory when it leaves one state to enter another (the Markov assumption). Although it is always possible to define a complete collection of states for any system, the Markov assumption may not be valid in some cases (e.g., if there are no free energy barriers between different regions and the movement is purely diffusive). Saturation properties expressed as the probability of multiply occupied states with an explicit number of ions inside the pore are not a consequence of the Markov assumption, but are shown here to follow directly from a statistical mechanical analysis. Although kinetic models are formulated in terms of transition rate constants, equilibrium properties do not rely on the Markov assumption. However, the form of $\mathcal{P}_n$ in Eq. 45 does not necessarily imply that an ion binds at a specific location inside the pore. The n-ion binding constants K_n are expressed as integrals over the whole pore region (see below), with no assumptions concerning specific binding sites. In addition, it should be noted that there is a hidden dependence on the ion concentration in the association constants K_n, despite the simple form of Eq. 45. This effect can be reduced by keeping the ionic strength of the solutions constant with impermeable ions while the concentration of a specific ion is varied.

The GA channel provides a useful example for examining the significance of the binding constants at the microscopic level. A cation-binding site is located near the entrance at each end of the dimer channel (Olah et al., 1991). At most, one of the binding sites is occupied at low concentrations of permeant ions, whereas the two sites may be occupied simultaneously at higher concentrations. There is no experimental evidence for a multiply occupied state with three ions. Analysis based on molecular dynamics simulations indicates that ^{13}C and ^{15}N solid-state NMR chemical shift anisotropy data (Smith et al., 1990; Tian et al., 1996) are consistent with a pair of Na^+-binding sites located ± 9.2 Å from the center of the channel (Woolf and Roux, 1997). Those positions correspond to the minima found in MD free energy calculations with Na^+ (Roux and Karplus, 1993). Studies of ^{13}C chemical shift changes upon Na^+ binding to GA channels incorporated into lipid vesicles were analyzed in terms of a tight and a weak binding constant, corresponding to singly and doubly sodium-occupied channels, which were estimated to be 67 M^{-1} and 1.7 M^{-1}, respectively (Jing et al., 1995). Another estimate for the singly occupied association constant, based on TI 205 NMR spectroscopy, is 31.6 M^{-1} (Hinton et al., 1988). Other estimates for the singly and doubly occupied equilibrium constant, obtained from an analysis of nonequilibrium ion flux data, are 7.34 M^{-1} and 0.25 K^{-1}, respectively (Becker et al., 1992).

The variations of the average NMR chemical shifts as a function of Na^+ concentration can be understood on the basis of Eqs. 8 and 45, because those measurements were performed at equilibrium. In the present formulation K_1 corresponds to the equilibrium association constant to load one ion into any of the two sites of the GA channel, whereas the equilibrium association constant for loading a second ion is $K_d = 2K_2/K_1$. The one-ion binding constant corresponds to a three-dimensional volume integral of the Boltzmann factor, $\exp[-(\mathcal{W}(\mathbf{r}_1) - \bar{\mu})]$. For simplicity we assume that the solutions are symmetrical and that $\bar{\mu}^{(I)} = \bar{\mu}^{(II)}$. In the case of the GA channel, the volume integral is dominated by the two local minima in the PMF near the channel entrance,

$$K_1 = \int_{\text{pore}} d\mathbf{r}_1 \, e^{-\beta[\mathcal{W}(\mathbf{r}_1) - \bar{\mu}]} \approx 2 \times \delta v_b \, e^{-\beta[\mathcal{W}_b - \bar{\mu}]}, \quad (46)$$

where $\delta v_b = \delta x_b \delta y_b \delta z_b$ is the volume corresponding to the fluctuations of the Na^+ ion in one binding site, and $[\mathcal{W}_b - \bar{\mu}]$ is the binding free energy of Na^+ relative to the bulk solution. MD of one Na^+ located in the binding site suggests that it spontaneously fluctuates between 8.5 and 10.5 Å along the channel axis (Woolf and Roux, 1997), whereas displacements off the channel axis are only on the order of 0.5 Å (Woolf and Roux, unpublished results). This yields a microscopic binding volume δv_b of 0.5 $Å^3$ for Na^+ in the binding site of the GA channel. An estimate of the free energy of one Na^+ in the entrance of the GA channel relative to the bulk solution can be obtained from the measured one-ion association constant as $-k_B T \ln(K_1/2\delta v_b)$ (n.b.: the measured binding constants in M^{-1} must be divided by 6.02×10^{-4} to be converted into $Å^3$). This yields values of -6.9 kcal/mol (Jing et al., 1995), -6.5 kcal/mol (Hinton et al., 1988), and -5.6 kcal/mol (Becker et al., 1992) for the relative free energy $[\mathcal{W}_b - \bar{\mu}]$. Interestingly, the various estimates of the free energy are in good accord, despite the vast differences in methodology and experimental conditions. It should be noted that the volume factor in the definition of the absolute binding constant is often overlooked (see Gilson et al., 1997, for a recent discussion). In particular, the quantity $-k_B T \ln(K_1)$, with K_1 expressed in M^{-1}, does not correspond to a meaningful binding free energy. For example, this expression yields only a free energy of -2.5 kcal/mol with a binding constant of 67 M^{-1}. Following Eq. 44, the binding constant can also be calculated as a one-dimensional integral along the channel axis in terms of the free energy profile $w(x)$ and the cross-sectional area $S(x)$. According to Eq. 43, the cross-sectional area is related to the fluctuations perpendicular to the channel axis. The off-axis fluctuations of Na^+ in the GA channel are on the order of 0.5 Å (Woolf and Roux, unpublished results), which yields a cross-sectional area on the order of 0.25 $Å^2$. This value is much smaller than estimates of the pore diameter based on van der Waals radii (Smart et al., 1993), although the magnitude of the binding constant is not very sensitive to the precise value of the cross-sectional area.

Comparison of the binding constant for the doubly occupied channel to that of the singly occupied channel provides information about ion-ion repulsion between two Na^+ located in the binding sites (Roux et al., 1995). Assuming that the ion binding sites do not change their positions in the doubly occupied state, the expression $-k_B T \ln(2K_d/K_1)$ gives an estimate of the ion-ion interactions in the GA channel. In terms of the one-ion and two-ion PMF, the ion-ion interaction is $[\mathcal{W}(\mathbf{r}_1,\mathbf{r}_2) - \mathcal{W}(\mathbf{r}_1) - \mathcal{W}(\mathbf{r}_2)]$. Repulsion energies of $+1.6$ and $+1.9$ kcal/mol are estimated from the data of Becker et al. (1992) and Jing et al. (1995), respectively. A value of $+6$ kcal/mol was obtained from MD free energy calculations (Roux et al., 1995). The bare repulsion in vacuum for two ions separated by 19 Å (on the order of $+18$ kcal/mol) is significantly reduced in the channel environment. Interestingly, the thermodynamics of double occupancy is sensitive to the ionic species; e.g., it is relatively easier to load two K^+ than two Na^+, because of the single-file structure of the water molecules in the narrow GA channel (Roux et al., 1995). Such an effect cannot be understood on the basis of a structureless continuum model of the pore environment. Similar energetic and structural considerations are expected to be very important in the characterization of the KcsA K^+ channel, which can contain several K^+ simultaneously (Doyle et al., 1998).

The PMF and the membrane potential

Our analysis, which led to Eq. 37, demonstrates that the total PMF can be written as a power series in V, the transmembrane potential,

$$\mathcal{W}(\mathbf{r}_1) = \mathcal{W}^{(0)}(\mathbf{r}_1) + \mathcal{W}^{(1)}(\mathbf{r}_1) + \mathcal{W}^{(2)}(\mathbf{r}_1) + \cdots \quad (47)$$

The intrinsic ion-pore free energy, $\mathcal{W}^{(0)}(\mathbf{r}_1)$, is dominated by the interactions of the permeating ion with the channel and its water content. It corresponds to equilibrium conditions, with $V = 0$ and symmetrical concentration of permeant ions on both sides of the membrane. In addition, long-range electrostatic interactions with the solvent, counterions, and lipid headgroups give rise to reaction field, electroosmotic (Jordan et al., 1989), and interfacial dipolar polarization effects (Shinoda et al., 1998), which also contribute to $\mathcal{W}^{(0)}(\mathbf{r}_1)$. However, $\mathcal{W}^{(0)}(\mathbf{r}_1)$ is not voltage-dependent. Only the remaining terms, $\mathcal{W}^{(1)}(\mathbf{r}_1)$ and $\mathcal{W}^{(2)}(\mathbf{r}_1)$, are due to the membrane potential. The coupling between the pore content (the permeating ion, the channel, and the water molecules inside the channel) and the transmembrane potential is mostly electrostatic in nature. For this reason, it seems reasonable that a continuum electrostatic description based on Eqs. 27 and 29 can be valid. In contrast, the microscopic interactions giving rise to the intrinsic ion-pore PMF arise from the quantum mechanical Born-Oppenheimer energy

surface of the molecular system, which includes complex contributions such as the influence of the charge distribution, core-core repulsion, London dispersion, induction, polarization, and charge transfer (Claverie, 1978). Molecular mechanical potential functions, such as AMBER (Cornell et al., 1995), CHARMM PARAM22 (MacKerell et al., 1998), or OPLS (Jorgensen et al., 1996), are aimed at reproducing the main features of this energy surface by using simple classical functional terms (Brooks et al., 1988). Furthermore, the intrinsic ion-pore PMF, $\mathcal{W}^{(0)}(\mathbf{r}_1)$, corresponds to a thermal average for the molecular system, in which the structural flexibility of the channel is expected to play an essential role. In particular, it has been shown that the PMF of Na^+ along the axis of the GA channel results from a complex interplay of water-water, water-channel, and channel-channel hydrogen bonding interactions (Roux and Karplus, 1991a). Therefore, although a continuum electrostatic representation for calculating $\mathcal{W}^{(0)}(\mathbf{r}_1)$ may be useful in the case of large wide channels, there is no reason to believe a priori that this is a valid approximation in the case of narrow channels, in which the ions and the water molecules are configured in single file.

Similar observations can be made concerning multiply occupied channels. The *n*-ion intrinsic PMF, $\mathcal{W}^{(0)}(\mathbf{r}_1, \ldots, \mathbf{r}_n)$, is a nonadditive many-body free energy potential. It is likely that pairwise additivity of ion-ion interactions is not a good approximation in the confined environment of a narrow pore. For example, MD free energy calculations showed that the double occupancy of the GA channel is sensitive to the ionic species, despite the fact that the binding sites are almost 20 Å apart (Roux et al., 1995). It is relatively straightforward to extend the present analysis to obtain the first-order contribution to the PMF in the case of a multiply occupied state with *n* ions inside the pore. Following arguments similar to those that led to Eq. 38, we write

$$\begin{aligned}\mathcal{W}^{(1)}&(\mathbf{r}_1, \cdots \mathbf{r}_n)\\ &= \langle \Delta\mathcal{F} \rangle_{(\mathcal{F}_{sym};\mathbf{r}_1, \cdots \mathbf{r}_1)} - \langle \Delta\mathcal{F} \rangle_{(\mathcal{F}^*_{sym})} = V\sum_{p=1}^{n} q_p \phi_{mp}(\mathbf{r}_p)\\ &+ V\left[\left\langle \sum_{p>n} q_p \phi_{mp}(\mathbf{r}_p) \right\rangle_{(\mathcal{F}_{sym};\mathbf{r}_1, \cdots \mathbf{r}_n)} - \left\langle \sum_{p>n} q_p \phi_{mp}(\mathbf{r}_p) \right\rangle_{(\mathcal{F}^*_{sym})}\right],\end{aligned} \tag{48}$$

where particles 1 to *n* are permeant ions and particles with $p > n$ are the remaining charges in the pore region (e.g., channel and water molecules). Because the transmembrane field $\phi_{mp}(\mathbf{r})$ is calculated with Eq. 29, in which the charges of the pore regions are not involved, it follows that the mathematical form of $\mathcal{W}^{(1)}(\mathbf{r}_1, \ldots, \mathbf{r}_n)$ is unchanged, regardless of the number of ions inside the pore region. To lowest order in *V*, the coupling to the membrane potential is the direct interactions of the changes in the pore region with the transmembrane field $\phi_{mp}(\mathbf{r})$. An explicit atomic representation of the channel and the water molecules inside the pore is required to calculate the contribution from the membrane potential. The total coupling to a transmembrane potential cannot be obtained with a continuum representation of the solvent because the average charge distribution in the pore region, with and without an ion present, is important. Therefore, despite its apparent simplicity, the first-order contribution $\mathcal{W}^{(1)}$ is also a many-body nonadditive function.

Linearity of the transmembrane potential

Our analysis shows that the transmembrane potential can be calculated on the basis of Eq. 29. To linear order in *V*, its dominant effect is to act on the ion, channel, and water molecules located in the pore region, as described by Eq. 38. If the local geometry of the channel-membrane system is approximately planar (e.g., the pore and the vestibule are relatively narrow and the length of the pore does not exceed the thickness of the membrane), it is possible that the field ϕ_{mp} in the pore region may be approximately linear along the channel axis over some length *L*. To examine the spatial dependence of the transmembrane field, we consider the case of the GA channel. For the purpose of the calculation, a typical atomic configuration of the channel embedded in an explicit lipid bilayer is used. In practice, an average over a number of configurations (channel and membrane) should be performed, although no large variations are expected in the present case. The model incorporates one GA channel and 10 single-file water molecules embedded in a lipid bilayer constituted by 100 dimyristoylphosphatidylcholine (DMPC) molecules (50 molecules in the upper and lower leaflet, respectively). In the model, the bilayer extends in the *y*, *z* plane, and the GA channel is located at the origin of the system with the pore parallel to the *x* axis. The construction procedure for generating the model is described briefly in Fig. 2, although the details are not expected to be very important for the purpose of the present calculations.

The electrostatic transmembrane field $\phi_{mp}(\mathbf{r})$ is calculated by solving Eq. 29 numerically as described previously (Roux, 1997). In the calculation, the channel and the membrane are represented in full atomic detail, and the solution is represented as continuous media. The solution is modeled by a uniform dielectric constant of 80 with a salt concentration of 150 mM. Values of one and zero are assigned to the dielectric constant and the Debye screening factor, respectively, in the interior of all explicit atoms (protein and lipids). The same values are imposed for the pore region because the counterions from the bulk as well as solvent molecules, other than the 10 explicitly represented single-file water molecules, are excluded from the pore region because of the Heaviside step function $H_p(\mathbf{X}_r)$ in the integral of Eq. 26. We assume that the pore region is a cylindrical region of 5-Å radius extending from −12.5 Å to +12.5 Å along the *x* axis. The cylinder corresponds to the most cation-selective part of the GA channel. This choice is consistent with free energy calculations that have shown that Cl^- anions cannot penetrate into the GA channel because their presence is energetically unfavorable, due to the

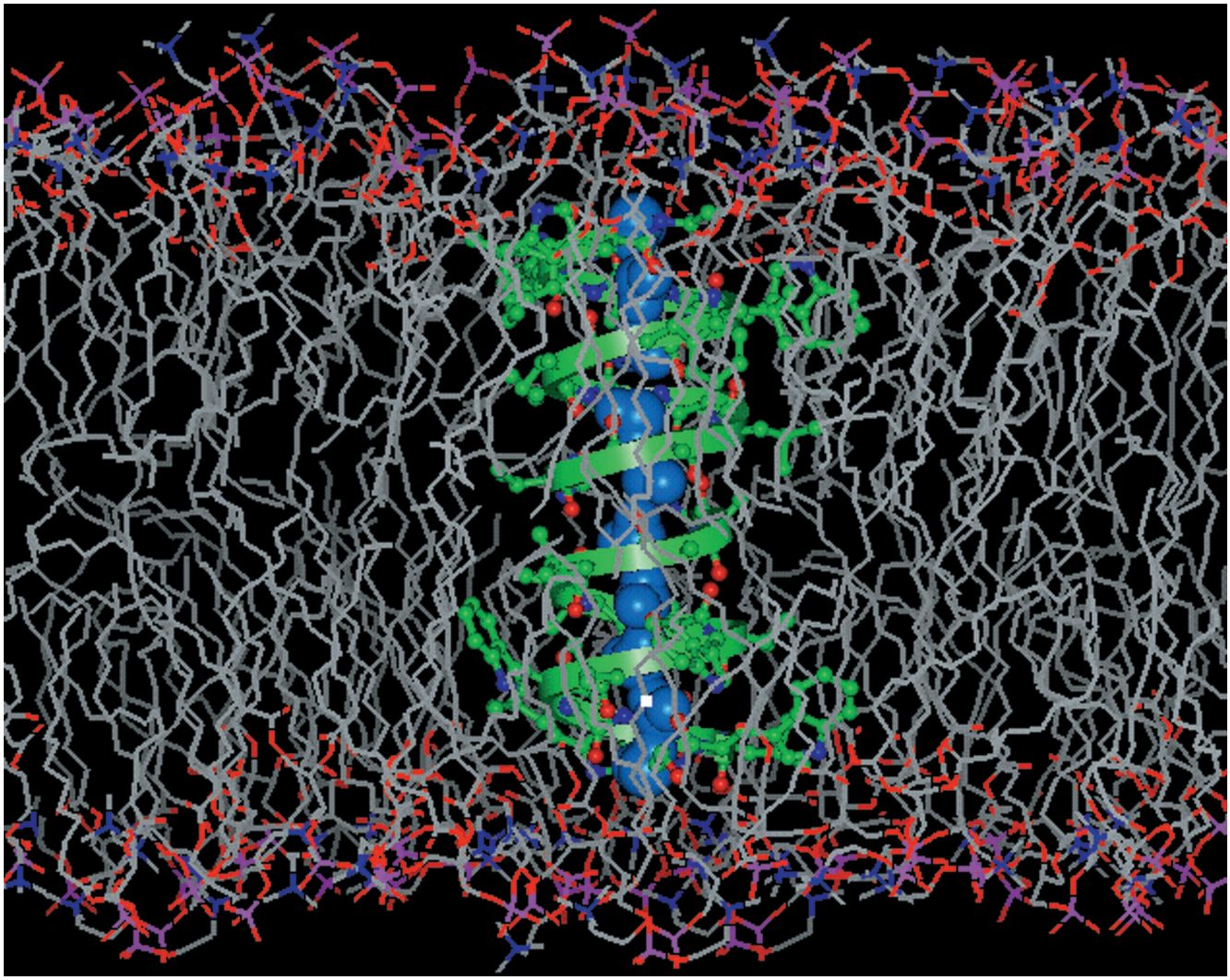

FIGURE 2 Picture of the atomic configuration of the GA in a DMPC bilayer used to calculate the transmembrane field ϕ_{mp}. The model incorporates one GA channel, a lipid bilayer constituted by 100 DMPC molecules (50 molecules in the upper and lower leaflets, respectively), and 10 single-file water molecules. The model was assembled using an equilibrated configuration of a GA:DMPC system (Woolf and Roux, 1996). The initial configuration with one GA and 41 DMPC was extended to yield a large patch of membrane by adding 59 preequilibrated DMPC molecules, using a methodology developed previously (Woolf and Roux, 1994, 1996). The final configuration was refined with energy minimization. Periodic boundary conditions were applied in the *yz* directions to simulate an infinite bilayer; the dimension of the central unit was chosen to be 58 Å, to correspond roughly to the cross-sectional area of one GA and 50 DMPC molecules (Woolf and Roux, 1996).

charge asymmetry of the backbone structure (Roux, 1996). In practice, the choice of the radius is not critical, as long as the interior of the pore is included (see Fig. 1).

The calculated transmembrane potential field along the *x* axis is shown in Fig. 3 for different *y*, *z* positions near the center of the pore region. The field is clearly linear, although there are small deviations near the entrance of the channel. In part, this is due to the fact that the thickness of a DMPC membrane matches quite well the length of the GA channel. Furthermore, the high dielectric constant of water and the screening due to the mobile ions in the bulk solution damp out the electric field everywhere except in the membrane and pore region. Fig. 3 is similar to the results obtained previously by Jordan et al. (1989) in the case of a cylindrical pore. They arrived at essentially the same conclusion: "the presence of electrolyte significantly affects the voltage profile due to an applied potential, substantially compressing the electric field to the immediate vicinity of the pore itself" (Jordan et al., 1989).

The fraction of the field felt by a cation located in the entrance binding site at -9.5 Å is on the order of 15%, in good agreement with previous estimates based on analysis data (Becker et al., 1992; Busath and Szabo, 1988). The calculation suggests that the form

$$\phi_{mp}(\mathbf{r}) \approx \begin{cases} 0 & \text{if } x < -L/2 \\ (x/L + 1/2) & \text{if } -L/2 < x < +L/2 \\ 1 & \text{if } x > +L/2 \end{cases} \qquad (49)$$

with a length L of 25 Å is reasonable for the GA channel. However, it should be stressed that the observed linearity of the transmembrane field ϕ_{mp} implies nothing about the

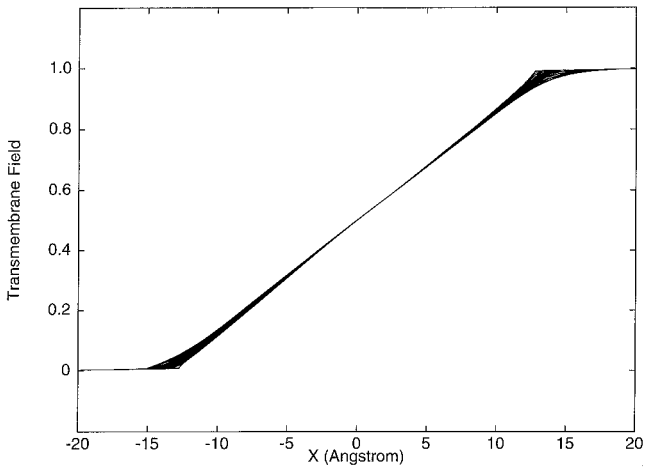

FIGURE 3 Calculated transmembrane field ϕ_{mp} along the x axis plotted for several values of y, z near the center of the pore. The results were obtained by solving Eq. 29 numerically. The field along the x axis for y and z between -2.5 and $+2.5$ Å are shown. The transmembrane voltage is given by $V \times \phi_{mp}$. The electrostatic problem was mapped onto a discretized cubic grid of $160 \times 116 \times 116$ points in the x, y, z directions with a grid spacing of 0.5 Å and periodic boundary condition in the membrane plane. The dielectric boundary between the molecules and the solvent was constructed based on a set of atomic Born radii derived from the average solvent radial charge distribution functions around the 20 amino acids in molecular dynamics simulations with explicit water molecules (Nina et al., 1997). The numerical calculations were carried out using a standard relaxation algorithm (Klapper et al., 1986; Warwicker and Watson, 1982). The continuum electrostatic calculations were performed using a modified version of the modified Poisson-Boltzmann Eq. 27 implemented in the PBEQ module (Beglov, Im, and Roux, unpublished work) of the biomolecular program CHARMM (Brooks et al., 1983).

linearity of the intrinsic PMF $\mathcal{W}^{(0)}(\mathbf{r}_1)$; i.e., it is a necessary but insufficient condition, because the true voltage coupling to the ion's motion is via the PMF. Following from Eq. 49, the PMF is

$$\mathcal{W}(\mathbf{r}_1) = \mathcal{W}^{(0)}(\mathbf{r}_1) + \left[q_1\left(x_1 + \frac{L}{2}\right) + \overline{\delta D_x(\mathbf{r}_1)} \right] \left(\frac{V}{L}\right) - \frac{1}{2k_BT}\left[\overline{\delta D_x^2(\mathbf{r}_1)}\right]\left(\frac{V}{L}\right)^2, \quad (50)$$

where $\overline{\delta D_x(\mathbf{r}_1)}$ and $\overline{\delta D_x^2(\mathbf{r}_1)}$ are, respectively, the average and fluctuations of the x-component of the dipole moment of the channel and water content of the pore with the ion at $\mathbf{r}_1$ relative to the unoccupied channel.

The first-order contribution can be written as $\mathcal{W}^{(1)} = q_1 x_{elec} V/L$, where $x_{elec} = x_1 + L/2 + \overline{\delta D_x(\mathbf{r}_1)}/q_1$ plays the role of an effective "electric distance" (Hille, 1992). Deviations of the electric distance from the simple geometric position $[x_1 + L/2]$ depend on the magnitude of $\delta D_x(\mathbf{r}_1)$, the variation of the dipole moment of the channel and water in the pore region. In the case of the GA channel, the β-helical structure does not undergo large conformational changes in the presence of an ion (Tian et al., 1996; Woolf and Roux, 1997). In such a situation, the variations in the dipole moment are expected to be dominated by the anisotropic orientational polarization of water molecules. Detailed MD studies indicate that the orientation of the 10 water molecules disposed in single file along the channel axis is significantly affected by the presence of an ion (Roux et al., 1995; Roux and Karplus, 1993; Woolf and Roux, 1997); one Na^+ located near the entrance of the channel is sufficient to roughly orient six of the single-file water molecules, with their oxygen pointing toward the ion. In the absence of ions the single-file water molecules form hydrogen-bonded chains with their dipole parallel to the channel axis (Chiu et al., 1991; Mackay et al., 1983), although a symmetrical arrangement has also been observed (Roux et al., 1995). The maximum dipole moment of a single file oriented along the x axis in the absence of an ion is $\sim 3\ e$Å (Pomes and Roux, 1998). By symmetry, the orientation of the whole single file is reversed when the ion moves from one end of the channel to the other. Correspondingly, the orientation of the single-file water molecules affects the apparent electric distance by 3 Å toward the center of the channel. For an arbitrary ion position x_1, the average orientational polarization of the single-file water molecules is expected to take intermediate values, although there are small deviations (Roux and Karplus, 1991a).

The second-order contribution of the perturbation involves the fluctuations in the x component of the total electric dipole of the channel and the m internal water molecules while the ion is fixed at $\mathbf{r}_1$. In the case of the GA channel, it may be expected that the fluctuations in the dipole moment of the single-file water molecules are significantly reduced when the ion is in the pore (i.e., the presence of an ion "freezes" the single file). To estimate the importance of this contribution, we assume that the 10 single-file water molecules can fluctuate between two configurations with maximum dipole D_{max} in the absence of an ion in the channel. Accordingly, the rms fluctuations of the total dipole associated with the single file are $D_{max}\sqrt{2}$. For a membrane field corresponding to a change of 100 mV over 25 Å, the corresponding second-order energy contribution is $+0.36$ kcal/mol, opposing the binding of the ion in the pore. The magnitude of the second-order contribution is relatively modest, suggesting that the first-order contribution is dominant. Nonetheless, because the magnitude of $\mathcal{W}^{(2)}$ is quadratic with the membrane potential V, it increases rapidly to $+0.80$ kcal/mol for a potential of 150 mV.

This analysis shows that the coupling between the ion position and the transmembrane potential involves all of the atomic charges located in the pore region. In particular, the orientational polarization of the water molecules along the channel axis plays an important role. Further MD studies suggest that the orientation of water molecules is also anisotropic in a variety of channel models (Breed et al., 1996). No preferential order of water dipoles was observed in a hydrophobic β-barrel, whereas the water molecules were observed to orient antiparallel to the helix dipole in a tetrameric polyalanine bundle. A simulation of the OmpF porin channel trimer in a lipid membrane shows that the water molecules are oriented perpendicular to the channel

axis (Tieleman and Berendsen, 1998). A simulation of a hexameric alamethicin bundle in a lipid membrane indicates that the water molecules are strongly oriented along the channel axis over a distance of 20 Å (Tieleman et al., 1999). Most of these calculations were performed in the absence of permeating ions. Further work will be necessary to characterize the orientation of the water molecules in the presence of a permeating ion in those channels.

CONCLUSION

We have developed a rigorous statistical mechanical theory of the equilibrium properties of ion channels. General expressions, valid for a system with an ideally selective channel in thermodynamic equilibrium, were obtained for the probability of multiply occupied states, the free energy PMF, the influence of the membrane potential, and the free energy profile along the channel axis. The main results are as follows:

- The equilibrium probabilities of occupancy of multiply occupied channels have the familiar algebraic form of saturation properties, which is obtained from kinetic models with discrete states of denumerable ion occupancy (although this does not prove the existence of specific binding sites).
- The total free energy profile of an ion along the channel axis can be separated into an intrinsic ion-pore free energy PMF, independent of the transmembrane potential, and other contributions that arise from the interfacial polarization.
- To linear order in the transmembrane potential V, the contribution from the membrane potential is expressed as the average interaction of all of the charges in the pore region with the membrane field when an ion is present in the channel minus the average interaction of all of the charges in the pore region with the membrane field when no ion is present.
- Higher order corrections are related to the fluctuations of the interaction of all of the charges in the pore region with the membrane field, with and without ion present in the channel.
- The contribution from the membrane potential can be calculated using a modified Poisson-Boltzmann theory as the interaction of the charges in the pore region with a field, in the absence of the charges in the pore.
- In the case of narrow channels such as the GA channel, the calculated transmembrane potential resulting from the interfacial polarization is shown to be fairly linear over a significant fraction of the permeation pathway.

It is useful to review the main steps that led to the conclusions concerning the linearity of the membrane potential to appreciate their scope and significance. Above all, the conclusion relies upon the observed linearity of the transmembrane field $\phi_{mp}(\mathbf{r})$. The present analysis shows that this function is approximately linear in the ion's position along the narrow GA channel axis, as described by Eq. 50 and shown in Fig. 3. The function $\phi_{mp}(\mathbf{r})$, calculated by solving numerically the modified PB Eq. 29 for the detailed atomic configuration of the GA in a DMPC membrane, is shown in Fig. 2. Clearly, it can reasonably be expected that the transmembrane field will be roughly linear for most narrow selective channels. However, linearity of the transmembrane potential function $\phi_{mp}(\mathbf{r})$ is a necessary but insufficient requirement. The true free energy coupling between the ion's position and the transmembrane voltage V is via the PMF, $\mathcal{W}(\mathbf{r}_1)$. A cumulant perturbation expansion was used to express the total PMF as a power series in V. The first-order free energy correction, $\mathcal{W}^{(1)}(\mathbf{r}_1)$, which depends on the average charge distribution in the pore, is linear in V. In the case of the narrow GA channel, $\mathcal{W}^{(1)}(\mathbf{r}_1)$ is quite linear in the ion's position x_1 along the channel axis. The linearity in V also depends on the magnitude of the second-order correction, $\mathcal{W}^{(2)}(\mathbf{r}_1)$, which is quadratic in V. In the case of the GA channel, we showed that the second-order correction, which depends on the fluctuations of the charge in the pore, should be roughly negligible for V smaller than 150 mV.

In conclusion, our analysis indicates that the free energy profile is linear with respect to both V, the net transmembrane potential, and x_1, the ion's position along the channel axis, which is a reasonable approximation in the case of the narrow cation-selective GA channel. This provides some support for the constant membrane potential field approximation, a concept that has played a central role in the interpretation of flux data based on traditional models of ion permeation (Becker et al., 1992; Läuger, 1973; McGill and Schumaker, 1996). In part, this may explain why the position of the Na^+-binding sites in the GA channel dimer deduced from ion flux data using phenomenological models is in such excellent agreement with the results from ^{15}N and ^{13}C solid-state NMR chemical shift anisotropy (Smith et al., 1990; Tian et al., 1996; Woolf and Roux, 1997). However, the validity of the constant membrane potential field approximation depends clearly on specific details about a channel structure and dynamics and, for example, about how the average charge distribution and fluctuations are reflected in the first- and second-order free energy contributions. In practice, MD studies based on atomic models are necessary to examine the validity of such an approximation at the microscopic level.

A key ingredient for the formal development of the present theory is the concept of a pore region from which all ions other than the permeating ions are excluded. This is clearly an idealization. In reality, the presence of impermeable ions inside real channels is energetically unfavorable, but not absolutely forbidden. Although the concept of an ideally selective pore region may appear to be somewhat arbitrary, it represents a direct extension of the perfectly semipermeable membrane that is invoked in the derivation of the Nernst membrane potential (Hille, 1992). An equilibrium situation with asymmetrical solutions cannot exist unless the membrane-channel system is perfectly imperme-

able to all but one ionic species. To proceed further, one must therefore replace the finite (though significant) selectivity of the pore region of a real channel into an ideally selective pore region. The pore region should be chosen, on energetic and structural grounds, to correspond to the most selective region of an ion channel. In the case of the narrow cation-specific GA channel, the choice of a pore region is relatively straightforward: anions cannot penetrate inside the channel because their interaction with the backbone is energetically unfavorable (Roux, 1996). Similarly, a potassium-selective pore region can be defined for the KcsA channel (Doyle et al., 1998), and it may reasonably be expected that this will be the case for other selective biological channels. For wide unselective channels it may be difficult, or even impossible, to identify a well-defined pore region. Nonetheless, it should emphasized that the concept of an ideally selective pore region is not needed if one's goal is simply to formulate a statistical mechanical theory describing the equilibrium state of an ion channel embedded in a membrane with symmetrical solutions and no membrane potential. Such a theory would yield similar expressions for the probabilities of multiply occupied states and *n*-ion PMFs, which could be used to characterize the selectivity of an ion channel without making ad hoc assumptions about a pore region.

Although the present paper was only concerned with equilibrium properties, it is hoped that the formulation will provide a sound physical basis for assessing nonequilibrium theories of ion fluxes in channels. One may anticipate that a reasonable formulation of nonequilibrium properties following from the present work should have the following features: a multiion diffusion-like theory, derived from fundamental considerations about fluctuation-dissipation, and constructed in such a way that the ion distribution relaxes back to the configurational probabilities determined by the Grand Canonical Partition function of Eq. 22. Clearly, the construction of a nonequilibrium theory will require careful consideration.

In future work, the present theory will be used to examine the validity of the assumptions upon which current kinetic (Läuger, 1973) and continuous electrodiffusion models (Chen et al., 1997; Kurnikova et al., 1999; McGill and Schumaker, 1996) are established, and to investigate multiple-ion equilibrium occupancy effects in the GA channel (Ketchem et al., 1997), OmpF porin (Cowan et al., 1992), and KcsA K^+ channel (Doyle et al., 1998).

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