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# A kinetic role for ionizable sites in membrane channel proteins

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**Abstract.** Electrically charged residues in a membrane channel protein will certainly have a direct effect upon its gating and selectivity if they are near the channel pore. It is customary to regard the charged state of such residues as a fixed feature of the channel. In this paper it is argued that far from being fixed, the charged state of ionizable residues near the pore will very probably change rapidly in response to the channel opening and to ions passing through it. Calculations are presented using simple models which demonstrate that changes in the dielectric environment and changes in the distances to other charged groups resulting from channel opening can shift the effective pK values of the sites by 3 or 4 units leading to switching of its charged state. Examples are given of how this time dependent charge state of ionizable residues may play an important role in the functioning of channels. Also, by considering the influence of the electric field due to the mobile ion upon the charge state of a residue in the channel wall, it is shown that a channel lined with acid residues may very effectively block the passage of cations while allowing the passage of anions.

**Key words:** Ion channel, membrane receptor channel, protein ionizable sites, dielectric environment, charged amino acid residues, ion transport kinetic

## Introduction

There can be little doubt that the charged amino acid side chains of the channel protein play an important role in the gating and selectively of membrane ion-channels. Certainly the substitution of particular charged residues in the cloned nicotinic acetylcholine receptor by uncharged or oppositely charged residues has a marked effect upon its operation (Imoto et al. 1988). However, when attempting to model the operation of such channels, it has been assumed that these residues remain constantly charged throughout the functioning of the channel. In this paper it is shown that this is most unlikely to be so and that alterations in the positions of other charges in the vicinity of a charged residue and changes in its dielectric environment when the channel is opening or shutting, will affect greatly the probability that the residue remains charged.

A recent development that reinforces the need to examine this aspect of channel behaviour is the measurement of the volume of aqueous pore that is created when a channel opens. Osmotic stress experiments indicate an opening pore volume as large as 1,300 $Å^3$ for the potassium channel of the squid giant axon (Bezanilla et al. 1986) and a pore volume of about $2-4 \cdot 10^4$ $Å^3$ for the mitochondrial voltage dependent anion channel (Zimmerberg and Parsegian 1986). Such large changes in volume indicate large movements of major portions of the channel protein such as has been postulated in the models of Unwin and Ennis (1984), Doring and Colombini (1985) or Edmonds (1985). The creation of such a large and highly polarizable core within the channel protein is a major perturbation of the dielectric environment of a charged residue in the channel protein.

The aim of this paper is to illustrate in as transparent and general manner as possible the electrical effects discussed. To this end only simple models are employed. Within the limitations of the assumptions of the models, the solutions are exact but as mimics of real biological material they must be deficient in that they assume homogenious material of fixed dielectric constant and exact geometric shapes. By using more complex models it may be possible to perform more accurate electrostatic calculations (Rogers 1986; Matthew et al. 1985). For example it is clearly preferable to treat the immediate environment of a charged group in terms of individual polarizable molecules rather than as a homogeneous dielectric block (Warshel and Russel 1984). This could be particularly important for charged sites in channel proteins if the water molecules within the pore of a channel are elec-

trically ordered (Edmonds 1984). However, despite their limitations, simple models have been chosen as best suited to illustrate the general effects treated in this paper. The effects discussed are large and even these simple models probably represent a more accurate view of the real situation that has hitherto been obtained by ignoring such effects entirely.

### The ionizable site

The kinetic diagram for an acid site, such as COOH, that becomes charged ($COO^-$) when the proton is detatched is sketched in Fig. 1 a. The probability that the site is charged is denoted by $n$. The rate constant for binding a proton $\alpha$ is proportional to $P(v)$ the number of $H_3O^+$ molecules in an access volume $v$ surrounding the site. This is given by

$$P(v) = v[H_3O^+] = v N\, 10^{-pH},$$

where $N$ is the molecular concentration of water. If a voltage difference $V$ exists between the site surface and the bulk water then $P(v)$ must be multiplied by the appropriate Boltzmann factor to become

$$P(v) = v N\, 10^{-pH} \exp(-q V/k T),$$

where $q$ is the proton charge. The rate constants $\alpha$ and $\beta$ then become

$$\alpha = [R \exp(-B/k T)]\, v N\, 10^{-pH} \exp(-q V/k T)$$

and

$$\beta = [R \exp(-B'/k T)],$$

where $R$ is a rate constant, $B$ is the height of the energy barrier to be surmounted on making the transition between the states $|n\rangle$ and $|1-n\rangle$ and $B'$ is the barrier for the return transition. It is convenient to separate $V$ into $V_{site}$ due to the site itself and $V_{ext}$ due to external sources so that

$$V = V_{site} + V_{ext}\,.$$

If we define $pK_0$ as the pH at which the site has a 50% chance of being charged (i.e. $\alpha = \beta$) when $V_{ext}$ is zero, then we can write the ratio of the rate constants as

$$(\alpha/\beta) = 10^{(pK_0 - pH)} \exp(-q V_{ext}/k T)\,. \tag{1}$$

The probability that the site is charged is given by

$$n = 1/[1 + (\alpha/\beta)]\,. \tag{2}$$

Thus Eqs. (1) and (2) show that if $pH = pK_0$ and $V_{ext} = 0$ then $(\alpha/\beta) = 1$ and $n = 0.5$ as required. These equations also show that as far as $n$ is concerned a decrease in $pK_0 - pH$ of 2 units is exactly equivalent to an increase $\Delta V = +119.15$ mV in the external voltage at the site $V_{ext}$ because at room temperature

$$\exp(-q \Delta V/k T) = 10^{-2}\,. \tag{3}$$

Similarly, for a basic site which becomes positively charged by capturing a proton, the kinetic diagram is sketched in Fig. 1 b. Equation (2) remains true with the ratio of the rate constants defined in Fig. 1 b given by

$$(\alpha/\beta) = 10^{-(pK_0 - pH)} \exp(+q V_{ext}/k T)\,. \tag{4}$$

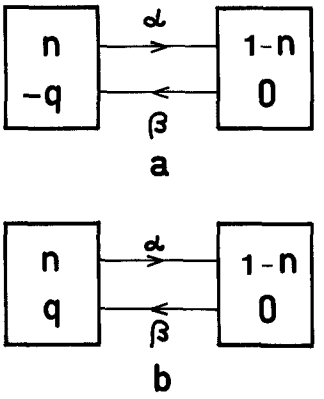


**Fig. 1. a** The kinetic diagram of an ionizable acid site with probability $n$ of having a charge $-q$ and $1-n$ of having no charge. **b** The similar diagram for an ionizable basic site

In the following three sections we will compute the effects on the voltage $V_{ext}$, and hence on $n$, at an ionizable acid site brought about by:

(*i*) changes in the dielectric environment in the absence of other charges,
(*ii*) changes in the proximity of other charged sites and
(*iii*) changes brought about by the passage of a mobile ion near to the site.

### The influence of the dielectric environment

In Fig. 2a is shown a univalent acid site, represented by a charged conducting sphere of charge $-q$ and radius $a$, at the plane interface between a semi-infinite slab of solid material with dielectric constant $\varepsilon_2$ bathed in a fluid of dielectric constant $\varepsilon_1$. In this configuration the voltage $V$ at position $F(Z, R)$ due to the site when charged may be found exactly by solution of the Laplace equation (Bleaney and Bleaney 1976). It is spherically symmetric and given by

$$V(Z, R) = \frac{-q}{2\pi\varepsilon_0(\varepsilon_1 + \varepsilon_2)} \left\{ \frac{1}{(Z^2 + R^2)^{1/2}} \right\}. \tag{5}$$

In Fig. 2b is shown the situation when a second semi-infinite and parallel solid slab also of dielectric constant $\varepsilon_2$ approaches the first slab. The voltage at $F(Z, R)$ is now axially symmetric about the $Z$-axis and may be obtained as a convergent sum using the method of images (Neumcke and Läuger 1969). It is given by

$$V(Z, R) = \frac{-q}{2\pi\varepsilon_0(\varepsilon_1 + \varepsilon_2)} \left\{ \frac{1}{(Z^2 + R^2)^{1/2}} \right. \tag{6}$$

$$\left. + \sum_{S=1}^{\infty} \frac{m^{2S-1}}{[R^2 + (2Sd - Z)^2]^{1/2}} + \sum_{S=1}^{\infty} \frac{m^{2S}}{[R^2 + (2Sd + Z)^2]^{1/2}} \right\},$$

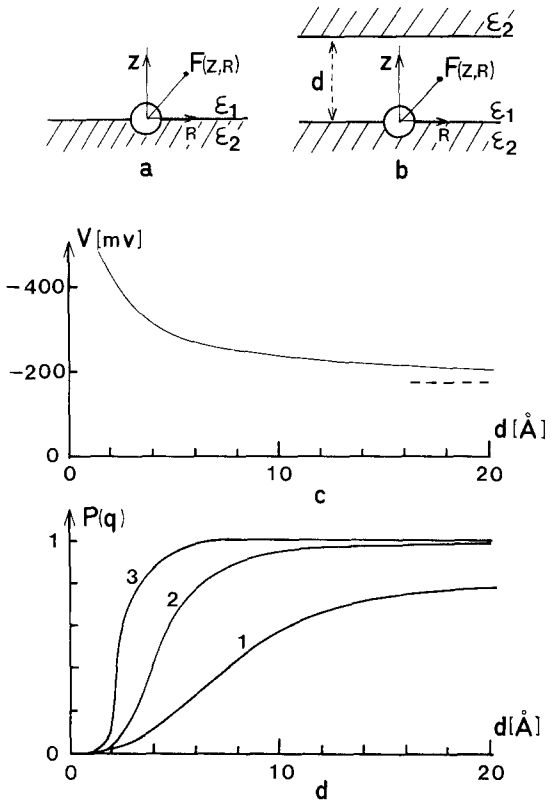


**Fig. 2.** **a** A conducting sphere of radius $a$ and charge $-q$ representing an ionizable acid site embedded in the interface between a plane solid slab of relative dielectric constant $\varepsilon_2$ bathed by a fluid of relative dielectric constant $\varepsilon_1$. **b** The same charged sphere when a second identical plane solid slab approaches to a distance $d$ from the first slab. **c** The voltage at position $F(Z, R)$ in Fig. 1 b as a function of $d$ where $Z=0$, $R=3$ Å, $\varepsilon_1=80$, $\varepsilon_2=2$ and $q$ is the proton charge. **d** Curves showing the probability that the site remains charged as a function of **d** with the parameters assumed above. The number beside each curve gives the value of $\mathrm{pH}-\mathrm{p}K_1$ where $\mathrm{p}K_1$ is defined as the value of pH at which the site has a 50% probability of being charged in the absence of the second solid slab

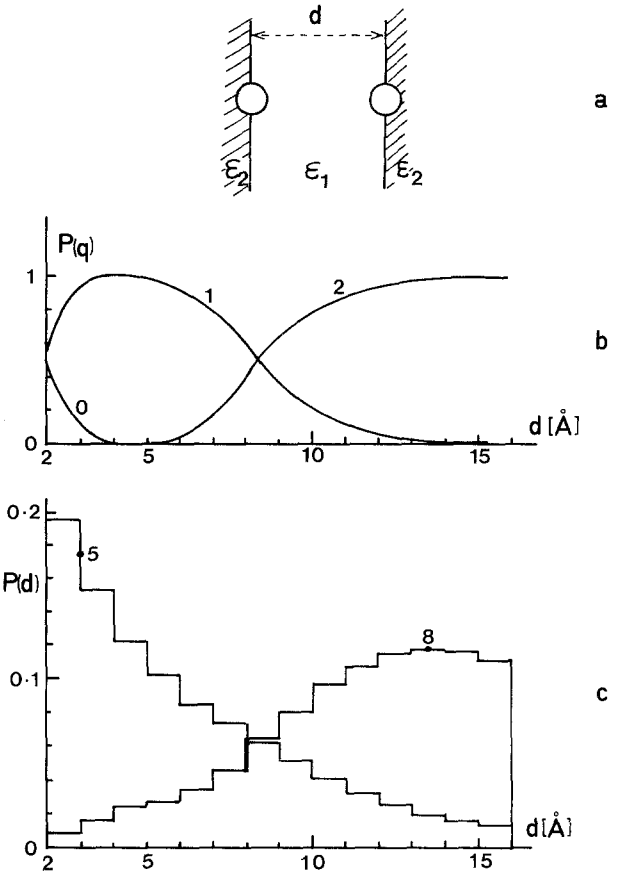


**Fig. 3.** **a** A representation of two acid sites, each embedded in a plane solid slab of relative dielectric constant $\varepsilon_2$, bathed in fluid of relative dielectric constant $\varepsilon_1$. The parallel slabs are separated by a distance $d$. **b** The probabilities, that in the configuration depicted in **a**, both sites are charged (2), one site only is charged (1) and that neither site is charged (0). The parameters assumed where $\varepsilon_1=80$, $\varepsilon_2=2$, $\mathrm{pH}-\mathrm{p}K_1=4$ and the radius of each site is 3 Å. **c** Histograms of the probability of finding $d$ in intervals of 1 Å with the parameters assumed in **b**. The $\mathrm{p}K_1$ for each site was taken as 4 and histograms are drawn for $\mathrm{pH}=5$ and $\mathrm{pH}=8$

where $m$ is the ratio $(\varepsilon_1-\varepsilon_2)/(\varepsilon_1+\varepsilon_2)$ and $d$ is the separation of the two slabs. The first and second summations represent the effects of charges induced at the second planar interface and at the original interface between the differing dielectrics. With $\varepsilon_1>\varepsilon_2$ the charges induced always have the same sign as the original charge and thus increase the magnitude of the potential $V$. The voltage at the position $Z=0$ and $R=3$ Å as a function of the separation $d$ of the two slabs is displayed in Fig. 2c for $\varepsilon_1=80$ representing water and $\varepsilon_2=2$ representing protein or lipid. For large $d$ the voltage is given by Eq. (5) with appropriate values of $Z$ and $R$ and this value is shown on Fig. 2c by a broken line. If all the space surrounding the site had dielectric constant $\varepsilon_2$ we would expect a voltage obtained by multiplying the prediction of Eq. (5) by the factor $(\varepsilon_1+\varepsilon_2)/(2\varepsilon_2)$. Figure 2c gives the prediction of Eq. (6) between these extremes and shows the rapid rise in the magnitude of $V$ at small $d$.

If a distance of 3 Å is assumed for the closest approach of the hydronium ion ($H_3O^+$) to the site, the voltages shown in Fig. 3c allow a prediction of the probability $P(q)=n$ that the site is charged using Eqs. (1) and (2). This is displayed as a function of $d$ for various values of $\mathrm{pH}-\mathrm{p}K_1$ in Fig. 2d. Here $\mathrm{p}K_1$ is defined as the effective $\mathrm{p}K$ of the site already embedded in the first slab but in the absence of the second slab, so that the changes in $P(q)$ shown are due only to changes in the proximity of the second slab. Thus even a site which at large $d$ has $\mathrm{pH}-\mathrm{p}K_1=3$, so that the probability of the site being uncharged is less than 1 part in 1,000, will lose its charge at small $d$.

## The influence of other charged groups

A simple model system to show the influence of a second charged group on the probability of a ionizable site being charged is sketched in Fig. 3a in which two identical acid sites, are embedded in the faces of parallel dielectric slabs, which can approach each other. Let us assume that at large $d$ both sites are negatively charged. As the slabs approach each other, both the dielectric effect discussed above and the voltage at one site due to the other will lead to increasing negative voltages at the two sites and thus a reduced probability of the sites remaining charged. To take a particular example I assume $\varepsilon_1 = 80$ and $\varepsilon_2 = 2$ as before and that $\mathrm{pH} - \mathrm{p}K_1 = 4$ so that at large $d$ each site has a probability of being uncharged of less than 1 part in 10,000. In Fig. 3b the computed probability that both sites are charged is displayed as a function of $d$ by the curve marked 2. The curves marked 1 and 0 show respectively the probability of finding a single site charged and no site charged. At small separations there is a good probability that both sites are uncharged because of the dielectric effect discussed above and the probability that both are charged at the same time is negligible at separations of less than about 6 Å.

This situation of like charges facing each other across a polarizable fluid has the interesting property that two very different stable states may be imagined. The first is at small $d$ when both sites are uncharged and thus there exists no repulsion between the sites. The second is at larger $d$ with both sites charged so that the repulsion between the charged sites is sufficient to keep the slabs apart. Positive feedback operates so that an increase in $d$ increases the probability of a further increase in $d$ and vice-versa. It is clear that two or more such charges facing each other could alone lead to channel gating due either to changes in pH or in the ambient voltage at the sites. This was investigated using a Monte Carlo computer model for the simplest configuration shown in Fig. 3a. A constant mechanical force was assumed to act in a direction to bring the slabs together with a magnitude equal to the electrostatic repulsion between the charged sites at a separation of 12 Å. The distance $d$ was assumed to vary between 2 and 16 Å. For the calculation this distance was divided into 100 steps. At any step the probabilities that none, one or both sites are charged may be calculated as in Fig. 3b. By comparing these probabilities with the output from a random number generator a particular charged state for the two groups could be determined. Knowing the charge states, the direct electrical repulsion and indirect dielectric repulsion are determined so that the probabilities of $d$ increasing by one step, decreasing by one step or remaining constant are determined. A random number generator is then consulted again to determine at that time which step if any is taken. In this manner the system may be allowed to evolve with time, constantly changing its state of charge and separation $d$ (one step at a time) dictated by the various room temperature Boltzmann statistical probabilities. Assuming a $\mathrm{p}K_1$ of 4 the average dwell times at various values of $d$ are shown in Fig. 3c for $\mathrm{pH} = 5$ and $\mathrm{pH} = 8$. As expected the calculations show that for the low pH there is a 79% chance that the gap between the slabs is less than 9 Å while at the higher pH there is a 75% chance that the gap remains greater than 9 Å. Changing the number of steps in the computation from 100 to 500 has little effect upon the results shown in Fig. 3c. The curve for $\mathrm{pH} = 8$, corresponding to $\mathrm{pH} - \mathrm{p}K_1 = 4$, results from the actual charge state probabilities displayed in Fig. 3b.

To imagine a channel opening mechanism based upon this effect would require a number, say 4, of parallel alpha-helices in a barrel-like configuration spanning the membrane as part of the channel protein. Each helix would have an acid residue at approximately the same distance along its length. At low values of $\mathrm{pH} - \mathrm{p}K_1$ the channel would be closed with most of the residues uncharged. If the pH were raised, the probability that two or more of the residues become charged increases, resulting in repulsive forces which tend to widen the channel. This widening in turn will co-operatively increase further the probability that the residues become charged which will lead to more opening. The "fully open" position will occur around the value of $d$ at which the membrane compressive forces equal the electrostatic repulsive forces.

As seen in Eq. (3) a change in the ambient voltage experienced at the acid sites is exactly equivalent to the change in pH in activating channel opening. A 2 unit increase in pH being the equivalent of a $+119$ mV change in the ambient voltage. For a voltage activated channel the extra voltage at the sites could be provided by the change in the charge structure of the channel which is measured (Keynes 1983) as gating charge transfer.

## The influence of transient ions

A major perturbation to any charged residues in the close vicinity of an ion channel is the electrical field from the ions which pass through the channel. To model this behaviour the configuration shown in Fig. 4a may be used. Again we have an ionizable site in the face of a solid block of dielectric constant $\varepsilon_2$ bathed by fluid of dielectric constant $\varepsilon_1$. An ion is constrained to move in a path of $N$ discrete steps situated at a distance $d$ from the solid slab. If the site bears a charge it will act as an effective electrostatic barrier to the passage of the ion whether they are of like or of

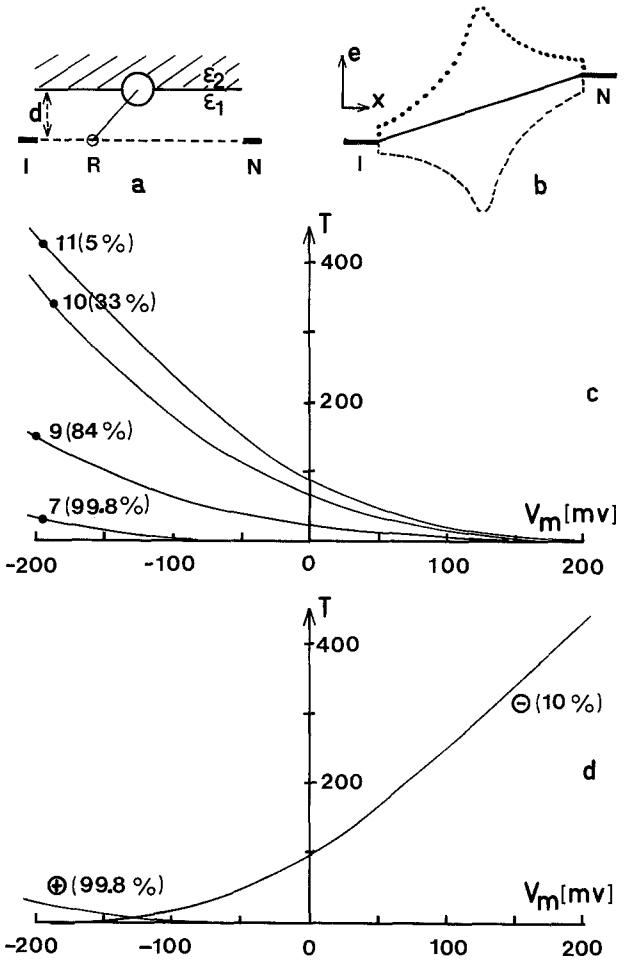


**Fig. 4.** **a** A diagram showing the path along which an ion is constrained to move in the vicinity of an acid site embedded in the plane face of a solid slab of relative dielectric constant $\varepsilon_2$ bathed by a fluid of relative dielectric constant $\varepsilon_1$. **b** The *full line* gives the assumed energy of (*i*) a cation when a positive potential $V_m$ is applied or (*ii*) an anion when a negative potential $V_m$ is applied. Step 1 is assumed to experience a voltage of $-V_m/2$ and step $N$ a voltage of $+V_m/2$. The *broken line* shows the energy experienced by a cation traversing the path when the acid site is charged. The *dotted line* shows the energy experienced by an anion traversing the path when the same acid site is charged. At sites 1 and $N$ the ion is assumed to be effectively screened from the electric field of the site. **c** The number of transits from left to right in a million time intervals of cations constrained to move along the path depicted in **a** using the model described in the text. The number beside each curve gives the assumed value of $pK_1$ and the percentage in brackets gives the average probability that the site bears a charge for $V_m = 0$ while a cation is located on the path during its transit. **d** A graph contrasting the number of passages per million time intervals from left to right along the path depicted in **a** of cations (+) and anions (−) for the same acid site with a $pK_1 = 7$. The pH is assumed to be 7 and the other parameters are as in **c**

opposite charge. However, the electric field of the ion changes the probability that the site remains charged, so that a complicated interplay of probabilities exists. Of particular interest is the fact that if the site is acid then a mobile positive ion will increase the probability that the site is charged while a negative ion will reduce this probability. As we shall see this leads to a kinetic selectivity based upon the sign of the mobile ion. Läuger (1985) has discussed in general terms the motion of ions through channels in which the barriers fluctuate stochastically with time. The present model is an extension of that case in which the very presence of the barrier directly depends upon the position of the ion within the channel.

Again the situation may be investigated by using a Monte Carlo computer model. For simplicity we will assume that the only forces acting are the electrostatic interaction between the ion and the site (when it is charged) and the force on the ion due to a voltage applied across the path representing the trans-membrane voltage. We will further assume that an ion occupying step 1 or step $N$ is effectively screened electrostatically from interaction with the site. Steps 1 and $N$ thus represent positions just outside the channel ends. In Fig. 4b the solid line shows the energy of an ion traversing the path when the site is not charged and only the membrane voltage acts. A voltage $V_m/2$ is assumed to act at step $N$ and $-V_m/2$ to act upon step 1 so that the membrane voltage at the site is zero. The solid line in Fig. 4b thus represents a positive membrane voltage for a mobile cation or a negative membrane voltage for an anion. The broken line in Fig. 4b shows the energy experienced by a cation when the acid site is charged and the dotted line the energy experienced by an anion. While an ion is traversing the path the electric field of its charge may change the charged state of the site and hence the energy profile of the ion path.

At each step along the path the electric field of the ion at the site may be calculated and hence, using Eqs. (1) and (2), the probability that the site is charged may be determined. Knowing this probability a random number generator is consulted to determine the charge state at that time. When the charge state of the site is known the energies of adjacent steps along the path are known, taking account of both the voltage due to the site (if charged) and that due to the membrane voltage $V_m$. Then the probabilities of taking a step to the right or to the left or remaining still are fixed and the random number generator is again consulted to find which move if any is made. Ions are introduced at step 1. The probability of entry to step 2 will of course depend upon the state of charge of the site as seen in Fig. 4b. Step one remains occupied by an ion whenever the rest of the path is empty. For a given set of parameters the system may be allowed to evolve with time and the number of ions which make the transition to step $N$ in a given number of time intervals may be determined. Only one ion is allowed on the path at any time and any ion that arrives at step $N$ is removed and replaced at step 1 once again. In

Fig. 4c are shown the number of successful transits $T$ in a million time intervals for positive ions as a function of the membrane voltage. Each curve corresponds to a particular value of $pK_1$ which is written beside that curve. The parameters assumed were $N=100$, $d=3$ Å, the path length from step number 1 to step number $N$ was 40 Å, pH = 7, $\varepsilon_1=40$ and $\varepsilon_2=2$. A reduced value of $\varepsilon_1$ was assumed to reflect the fact that the fluid was confined near a solid surface. Increasing the number of steps along the path from 100 to 500 does of course reduce the number of transits in a given number of time intervals but the shapes of the curves remains largely the same.

The probability that the site is charged depends upon the dwell time of transient ions in its vicinity and thus depends slightly upon the membrane voltage. The percentages given in brackets after the $pK_1$ value near each curve give the average probabilities for $V_m=0$ that the ionizable site is charged for that $pK_1$ and with a positive ion somewhere on the path. When the site is charged it acts as a considerable barrier to transit and Fig. 4c shows that large ion fluxes are possible only if there is an sizeable probability that the site is uncharged.

In Fig. 4d the number of transits for positive ions is contrasted with that for negative ions for the same acid site with $pK_1=7$. The reason for the large difference is that, although a charged site is a considerable barrier to ions of either sign (see Fig. 4b), the presence of the negative ion on the path reduces the probability of the site being charged to about 10% at $V_m=0$, while the positive ion ensures that the site is charged about 99.8% of the time.

It is normally assumed that a channel selective for cations will be lined by negative sites as this increases the Boltzmann probability of finding a cation in that vicinity over that in the bulk fluid. Indeed Fig. 4b shows that while the acid site is charged, cation entry is favoured although exit is hindered. However, as shown here, if the site has an effective $pK_1$ within the channel such that a transient ion may bring about a change in its charged state, then a channel that allows the transit of anions in either direction but prevents the transit of cations could well be lined with acid sites.

## Discussion

In this paper some of the consequences are considered of changes in the "effective $pK$" of an amino acid residue when changes occur in its electrical environment with particular reference to ion channels. It is argued that during the opening of a membrane channel and thus the creation of a considerable volume of electrically polarizable aqueous pore, the charge state of polarizable residues near the pore may vary rapidly. Thus, rather than being considered as static features of a channel structure, such sites may participate kinetically in channel function.

For example, it is well known that the opening of a channel may modulate the binding of ligands to the channel protein and thus modulate the action of pharmacological agents (Hille 1984). Clearly any change in the charge structure of the channel protein consequent upon opening as discussed here will markedly effect the binding of ligands, either directly through the changed external electric field if the ligand is charged, or indirectly by changing the geometry of the protein through changed internal electrostatic interaction.

Only the simplest cases have been considered here but clearly very complex behaviour is possible. For example, if Fig. 3a is taken to represent part of a channel held open by the repulsion of several acid sites which line it, then the passage of a positive ion through the channel will reinforce the charged state of these sites. However the presence of a negative ion could cause the sites to lose their charge so that the channel closes, thus exhibiting another kind of kinetic selectivity for the sign of the transient ion.

A particularly interesting extension of these ideas is evident when an acid and a basic residue near a channel are sufficiently close to interact strongly electrostatically. Each site, when charged, enhances the probability that the other is charged thus stabilizing the doubly charged state and reducing the probability of the charged state changing when the dielectric environment changes as illustrated in Fig. 2. Also the influence of the passage of an ion depends upon its direction of transit. These and other extensions of the concepts discussed here will be described in another paper.

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