Model for the Binding of the Inactivation N−Terminal to the Ion Pore of Shaker Potassium Channel: Both Electrostatic Attraction and Covalent Linkage Are Required for Rapid Inactivation

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A model is presented for relating the binding of the inactivation N-terminal to the ion pore of the *Shaker* potassium channel (ShB) to the bimolecular binding of the N-terminal peptide with the deletion mutant ShBΔ6−46. The binding site is modeled as a small patch on the surface of the channel protein, to which the N-terminal "inactivation ball" is tethered by a flexible linker. The potential energy due to electrostatic interactions between the channel and the N-terminal is $\beta U(r) = -Q\exp[-(r - a)/\lambda]/(1 + a/\lambda)r$, where a is the closest approach distance and λ is the screening length determined by the ionic strength. The probability density for the end-to-end vector of the flexible linker (with L residues) is taken from a previous study [Zhou, *J. Phys. Chem. B* **2001**, *105*, 6763] as $p(r) = (3/4\pi l_p bL)^{3/2}\exp(-3r^2/4l_p bL)(1-5l_p/4bL + ...)$. The intramolecular binding rate constant k_{on}^{in} of the intact ShB is related to the bimolecular binding rate constant k_{on}^{bi} via $k_{on}^{in} = k_{on}^{bi}p(a)/\int\exp[-\beta U(r)]p(r)dV$. The model rationalizes a number of important experimental observations. (1) The weaker ionic strength dependence of k_{on}^{in} is quantitatively reproduced by the relation between k_{on}^{in} and k_{on}^{bi}. (2) The linker length dependence of k_{on}^{in} (observed when the linker length is reduced by deletion and extended by insertion) is qualitatively predicted by the L dependence of $p(r)$. (3) The fact that $k_{off}^{in} = k_{off}^{bi}$ and both are insensitive to the change in ionic strength is due to the stereospecificity of the binding site. If the binding of the activation N-terminal were to occur in a bimolecular fashion, the millisecond inactivation time would have required the presence of the N-terminal at a concentration of 0.2 mM, even after considering the binding rate enhancement by the electrostatic attraction of the channel pore. The difficult task of maintaining such a high concentration underscores the importance of covalently linking the inactivation peptide to the ion channel.

Introduction

Voltage-gated ion channels open upon membrane depolarization and then close on a millisecond time scale. The rapid inactivation is crucial for the modulation of the firing frequency of the neurons. Inactivation occurs by the occlusion of the ion pore by an inactivation "ball" or "gate".[1] The ion channel is thus faced with the problem of having to affect rapid binding between the inactivation segment (IS) and the ion pore. If the binding were to occur in a bimolecular fashion and given that the stereospecificity of the binding between proteins limits the binding rate constant to 10^5 to 10^6 $M^{-1}s^{-1}$,[2] the millisecond inactivation time scale would require the maintenance of a population of the IS at a concentration of 1 to 10 mM. Long-range electrostatic interactions may enhance the binding rate constant,[3] but given the high ionic strength of the cytosol, one may expect an rate enhancement of just 10-fold. This still leaves the required supply of the IS at the mM range. Of course, the channels solve the problem of rapid inactivation by covalently linking the IS to the transmembrane region. This paper deals with the relation between bimolecular and intramolecular binding kinetics of a peptide segment to a protein receptor.

The paper builds on and extends earlier work on the relation between the thermodynamics of dimeric and single-chain protein folding.[4] We were able to relate the folding equilibrium constant K^d of a dimeric protein that has an interfacial hydrophobic core and unfold upon dissociation to the equilibrium constant K^s of a single-chain form obtained by the covalent linking of the C terminal of one subunit and the N terminal of the other. The result is

$$K^s/K^d = p(d_0) \tag{1}$$

where $p(r)$ is the probability density of the end-to-end vector of the flexible linker and d_0 is the end-to-end distance of the linker in the folded protein. For a flexible linker consisting of L residues, we showed that[5]

$$p(r) = (3/4\pi l_p l_c)^{3/2}\exp(-3r^2/4l_p l_c)(1 - 5l_p/4l_c + 2r^2/l_c^2 - 33r^4/80l_p l_c^3 - 79l_p^2/160l_c^2 - 329r^2 l_p/120l_c^3 + 6799r^4/1600l_c^4 - 3441r^6/2800l_p l_c^5 + 1089r^8/12\,800l_p^2 l_c^6) \tag{2}$$

where $l_c = bL$ with $b = 3.8$ Å and $l_p = 3$ Å. When the linker length l_c is much greater than both r and l_p, eq 2 reduces to $p(r) \approx (3/4\pi l_p l_c)^{3/2}$, which is the classical result of Jacobson and Stockmayer.[6]

Recently, we further showed that the DNA-binding of a single-chain protein consisting of two DNA-binding domains connected by a flexible linker can be related to the DNA-binding of the individual domains.[7] If the dissociation constants of the two domains for their respective half sites are K_A and K_B, then

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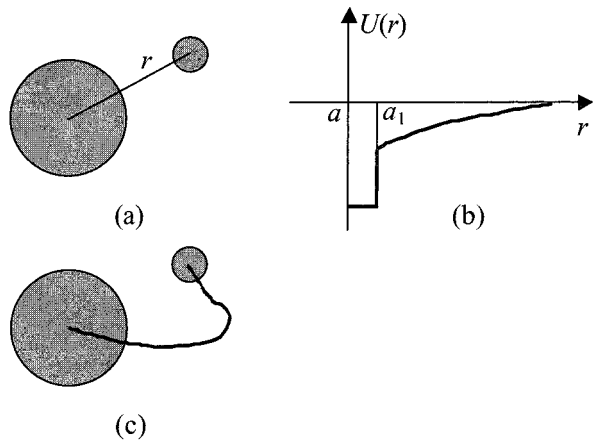

Figure 1. Binding of a spherical ligand to a spherical receptor. (a) Bimolecular binding. (b) The interaction potential. The potential well extends from the contact distance a to a_1, where the potential becomes a smooth function and slowly increases to zero. (c) Intramolecular binding when the ligand is tethered to the receptor by a flexible linker.

the dissociation constant K_{A-B} of the single-chain protein for the full binding site is

$$K_{A-B} = K_A K_B / p(d_0) \tag{3}$$

Here, we introduce a long-range interaction between the domains connected by the flexible linker. Our focus is to relate the intramolecular binding and unbinding rate constants (k_{on}^{in} and k_{off}^{in}) to the bimolecular binding and unbinding rate constants (k_{on}^{bi} and k_{off}^{bi}). In two situations, the relations are simple. The first consists of a spherical receptor and a spherical ligand interacting with a centrosymmetric potential. This simple model allows us to set up the framework of our approach. The second corresponds to the formation of a stereospecific ligand−receptor complex so that the binding site is small relative to the range of the interaction force. This situation is what is relevant for biological applications.

The theory developed is applied to the binding of the inactivation N-terminal to the ion pore of the *Shaker* potassium channel. We are able to rationalize a number of important experimental observations. (1) The weaker ionic strength dependence of k_{on}^{in} is quantitatively reproduced by the relation between k_{on}^{in} and k_{on}^{bi}. (2) The linker length dependence of k_{on}^{in} (observed when the linker length is reduced by deletion and extended by insertion) is qualitatively predicted by the L dependence of $p(r)$. (3) The fact that $k_{off}^{in} = k_{off}^{bi}$ and both are insensitive to the change in ionic strength is due to the stereospecificity of the binding site.

Theory

Binding of Spherical Ligand to Spherical Receptor. Consider the simple model shown in Figure 1a, where a spherical ligand interacts via a potential $U(r)$ with a spherical receptor. The potential has a deep well when the ligand is near contact with the receptor and immediately switches to a smooth function that slowly approaches zero (see Figure 1b). The ligand is considered bound whenever it falls to the potential well. The binding constant for the bimolecular reaction is[8−10]

$$K^{bi} = \int_a^{a_1} 4\pi r^2 \exp[-\beta U(r)]dr \tag{4}$$

where $\beta = (k_B T)^{-1}$. Unbinding occurs when the ligand escapes the potential well via diffusion. The rate constant can be calculated according to Kramers' theory.[11] The result is[10]

$$k_{off}^{bi} = D\{\int_a^{a_1} 4\pi r^2 \exp[-\beta U(r)]dr \int_{a_1}^{\infty} (4\pi r^2)^{-1} \exp[\beta U(r)]dr\}^{-1} \tag{5}$$

where D is the diffusion constant. The binding rate constant is thus

$$k_{on}^{bi} = K^{bi} k_{off}^{bi} = D\{\int_{a_1}^{\infty} (4\pi r^2)^{-1} \exp[\beta U(r)]dr\}^{-1} \tag{6}$$

which is the same as a result derived by Debye.[12]

We now consider the intramolecular binding shown in Figure 1c, where the ligand and receptor is connected by a flexible linker. If the end-to-end vector of the linker by itself has a probability density $p(r)$, then under the influence of the interaction between the ligand and the receptor, the probability density of the end-to-end vector is $p(r)\exp[-\beta U(r)]$. The equilibrium constant for the intramolecular binding is thus

$$K^{in} = \int_a^{a_1} 4\pi r^2 p(r) \exp[-\beta U(r)]dr / \int_{a_1}^{\infty} 4\pi r^2 p(r) \exp[-\beta U(r)]dr \tag{7}$$

Note that $p(r)$ is normalized such that $\int_a^{\infty} 4\pi r^2 p(r)dr = 1$. We are interested in cases where the width of the potential well is small. Then, between a and a_1, $p(r)$ is nearly a constant and one has

$$K^{in} = p(a)\int_a^{a_1} 4\pi r^2 \exp[-\beta U(r)]dr / \int_{a_1}^{\infty} 4\pi r^2 p(r) \exp[-\beta U(r)]dr \tag{8a}$$

$$= K^{bi} p(a) / \int_{a_1}^{\infty} 4\pi r^2 p(r) \exp[-\beta U(r)]dr \tag{8b}$$

If the long-range interaction potential is absent the integral in eq 8b reduces to $\int_{a_1}^{\infty} 4\pi r^2 p(r)dr$, which is nearly one because of the assumed closeness of a_1 to a and the normalization condition on $p(r)$. Then, we have $K^{in} = p(a_1)K^{bi}$. This result is equivalent to eq 1. Hence, eq 8b may be considered a generalization of eq 1 when long-range interactions between the binding partners are present.

We now move on to the kinetics of intramolecular binding. A flexible linker is equally unlikely to have both extremely small and extremely large end-to-end distances because the number of chain configurations diminishes in both limits, $4\pi r^2 p(r)$ will have a maximum at an intermediate value. The location $r = r_{max}$ of this maximum can be viewed as the equilibrium position of the unbound state. According to Kramers' theory, the unbinding rate constant is

$$k_{off}^{in} = D\{\int_a^{a_1} 4\pi r^2 p(r) \exp[-\beta U(r)]dr \int_{a_1}^{r_{max}} [4\pi r^2 p(r)]^{-1} \times \exp[\beta U(r)]dr\}^{-1} \tag{9}$$

Note that we have assumed that the linker does not change the diffusion constant of the ligand. The intramolecular binding rate constant is

$$k_{on}^{in} = K^{in} k_{off}^{in} = D\{\int_{a_1}^{\infty} 4\pi r^2 p(r) \times \exp[-\beta U(r)]dr \int_{a_1}^{r_{min}} [4\pi r^2 p(r)]^{-1} \exp[\beta U(r)]dr\}^{-1} \tag{10}$$

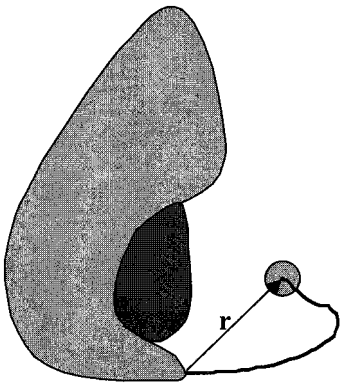

Figure 2. Stereospecific binding of ligand and receptor. The dark area represents the binding site on the surface of the receptor. The binding is intramolecular when the flexible linker is present and bimolecular when the linker is absent.

Stereospecific Binding. Now consider the stereospecific binding of a ligand to a receptor, as illustrated in Figure 2. By analogy to eq 4, the bimolecular equilibrium constant is

$$K^{bi} = \int_{\Gamma} \exp[-\beta U(\mathbf{r})] dV \tag{11}$$

where Γ denotes the binding site. The size of Γ is assumed to be small relative to the range of the interaction potential $U(\mathbf{r})$. In the derivations below, the situation where the interaction potential is absent [$U(\mathbf{r}) = 0$] will serve as an important reference. Equilibrium and rate constants when $U(\mathbf{r}) = 0$ will be signified by an additional superscript "0". In terms of K^{bi0}, we may write

$$K^{bi} = K^{bi0} < \exp[-\beta U(\mathbf{r})] > \tag{12}$$

where <...> means averaging over the binding site.

In a series of studies,[2,13,14] we showed that the bimolecular binding rate constant can be approximated by a relation analogous to eq 12

$$k_{on}{}^{bi} \approx k_{on}{}^{bi0} < \exp[-\beta U(\mathbf{r})] > \tag{13}$$

The last relation means that the unbinding rate constant is insensitive to the interaction potential

$$k_{off}{}^{bi} \approx k_{off}{}^{bi0} \tag{14}$$

This result can be rationalized by the following observations.[15] The unbinding rate constant is the inverse of the meantime it takes for the ligand to escape the binding site. When the binding site is small, this mean time will be very short. By the end of this short time, the ligand on average will not have moved very far from the binding site. If the interaction potential is smooth over this range, then the ligand would have moved in a nearly uniform potential. Hence, the presence of the potential does not play an important role as far as the escape from the binding site is concerned.

We have used eqs 13 and 14 to rationalize the disparate ionic strength dependences of the binding and unbinding rate constants observed on a wide range of associating proteins.[10] In fact, one of these systems is the binding of the peptide toxin Lq2 to the *Shaker* potassium channel. When the ionic strength was changed from 25 to 200 mM, the binding rate constant decreased by 41-fold; in contrast the unbinding rate constant increased by just 6-fold.[16]

When the ligand is tethered to the receptor by a flexible linker, the equilibrium constant becomes

$$K^{in} = \int_{\Gamma} p(r) \exp[-\beta U(\mathbf{r})] dV / \int' p(r) \exp[-\beta U(\mathbf{r})] dV \tag{15}$$

where the prime signifies that the integration is over the whole **r** space except for the region occupied by the binding site. Considering the small size of the binding site, we may write

$$K^{in} \approx p(d_0) \int_{\Gamma} \exp[-\beta U(\mathbf{r})] dV / \int' p(r) \exp[-\beta U(\mathbf{r})] dV \tag{16a}$$

$$= p(d_0) K^{bi} / \int' p(r) \exp[-\beta U(\mathbf{r})] dV \tag{16b}$$

where d_0 is the mean end-to-end distance of the tether when the ligand is bound. When the interaction potential is absent, eq 16b reduces to eq 1.

By the same argument that is used to justify eq 14, we assume that the tether will not significantly hinder the escape of the ligand from the binding site. Hence

$$k_{off}{}^{in} \approx k_{off}{}^{bi0} \approx k_{off}{}^{bi} \tag{17}$$

The intramolecular binding rate constant is thus

$$k_{on}{}^{in} = K^{in} k_{off}{}^{in} \approx k_{on}{}^{bi} p(d_0) / \int' p(r) \exp[-\beta U(\mathbf{r})] dV \tag{18}$$

Equations 16b, 17, and 18 are the main theoretical results of the paper. Note that when no interaction potential is present, we have

$$k_{on}{}^{in} \approx k_{on}{}^{bi} p(d_0) \tag{18a}$$

because of the normalization condition of $p(r)$. This result was suggested in a previous work.[4]

Separation of Short and Long-Range Interactions. It is important to recognize that, when a ligand approaches the binding site, it first encounters long-range electrostatic interactions. Once inside the binding site, it is stabilized by short-ranged van der Waals, hydrophobic, and hydrogen bonding interactions (long-range electrostatic interactions perhaps play a minor role here). The potential function depicted in Figure 1b is intended to illustrate the division of the short-range interactions inside the binding site (deep potential well between a and a_1) and the long-range electrostatic interaction outside (smooth potential for $r > a_1$). This division does not affect the main results for the present paper, eqs 16b, 17, and 18. We do need to note that the potential in eqs 16b and 18 refers to the long-range electrostatic interactions outside the binding site and $k_{off}{}^{bi0}$ in eq 17 refers to the bimolecular unbinding rate when the potential outside the binding site is turned off.

As noted previously,[10] in eq 13 $k_{on}{}^{bi}$ and $k_{on}{}^{bi0}$ refer to the diffusion-controlled bimolecular binding rate constants in the presence and absence of the long-range electrostatic interactions, and the average of the Boltzmann factor is over the outer edge of the binding site (corresponding to $r = a_1$ in Figure 1b).

Results

We now use the theory developed in the previous section to analyze experimental data on the binding of the inactivation N-terminal to the ion pore of the *Shaker* potassium channel (ShB). Our focus is the relations of the rate constants for the intramolecular binding in the intact ShB and the rate constants for the bimolecular binding of the N-terminal peptide with the deletion mutant ShBΔ6−46. The N-terminal peptide consists

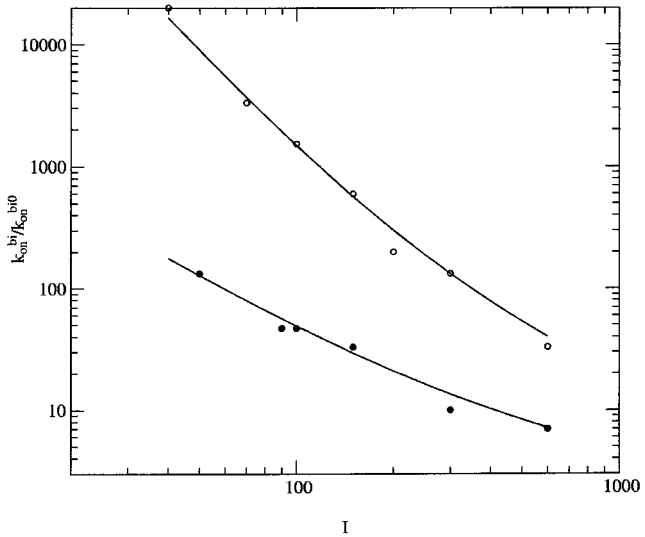

Figure 3. Ionic strength dependence of k_{on}^{bi} for the binding of the N-terminal peptide (solid circles) and the E12KD13K mutant (open circles) to ShBΔ6−46. The curves are the theoretical predictions by eq 20.

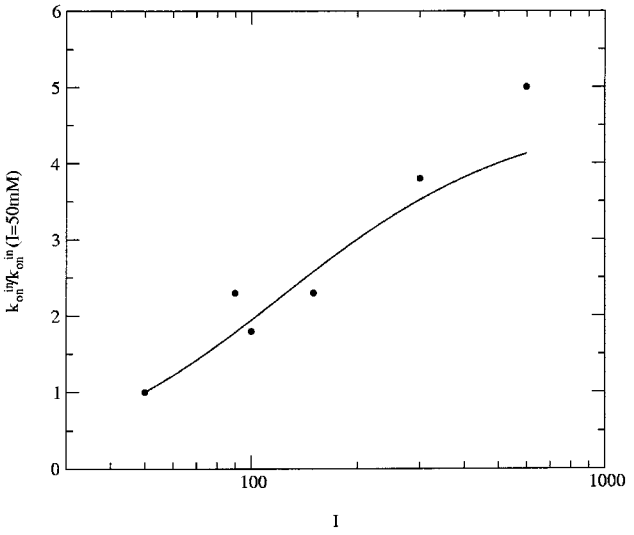

Figure 4. Ionic strength dependence of k_{on}^{in} for the intramolecular binding of the activation N-terminal to the ion pore of ShB. Circles are experimental results, whereas the curve is the theoretical prediction by eq 21.

of the first 20 residues of ShB.[17,18] The ShBΔ6−46 mutant is incapable of inactivation because of the disruption of the inactivation N-terminal.[17] Of particular interest is the ionic strength dependence of the rate constants.

Interaction Potential of ShB Inactivation N-Terminal and Ion Pore. A detailed electrostatic interaction potential can be calculated from the structure of the ion channel. We instead will resort to a simple model: the channel receptor will be described as a sphere with a uniform surface charge density and the N-terminal peptide will be described as a test charge. The interaction potential is then[19]

$$\beta U(r) = -Q\exp[-(r-a)/\lambda]/(1+a/\lambda)r \qquad (19)$$

where a is the radius of the receptor, and λ is the screening length related to the ionic strength I by $\lambda = 3.04I^{-1/2}$ at room temperature. If the binding site is modeled as a patch on the receptor sphere, then eq 13 predicts the following dependence on ionic strength for the bimolecular binding rate constant

$$k_{on}^{bi} \approx k_{on}^{bi0}\exp[Q/(1=a/\lambda)a] \qquad (20)$$

where k_{on}^{bi0} is to be identified with the rate constant at infinite ionic strength.

Figure 3 shows that the ionic strength dependence of the rate constant (k_{on}^{bi}) for the binding of the N-terminal peptide with the deletion mutant ShBΔ6−46, observed by Murrell-Lagnado and Aldrich,[20] is modeled well by eq 20 with $a = 20$ Å, $Q = 240$ and $k_{on}^{bi0} = 1.5 \times 10^5\ M^{-1}s^{-1}$. Moreover, the ionic strength dependence of k_{on}^{bi} for a mutant N-terminal peptide (E12KD13K) is modeled equally well by increasing Q to 450.

In sharp contrast to the 20-fold and 60-fold decreases of k_{on}^{bi} for the binding of the N-terminal peptide and the E12KD13K mutant to ShBΔ6−46, k_{off}^{bi} has a nearly constant value of ∼15 s^{-1} for both peptides when the ionic strength is increased from 50 to 600 mM.[20] This disparate ionic strength dependence between k_{on}^{bi} and k_{off}^{bi} is in line with results observed on a wide range of associating proteins,[10] including the binding of the peptide toxin Lq2 to ShB. It is exactly what is predicted by eq 14 and indicates that the binding of the N-terminal peptide is limited by the electrostatic enhanced diffusional encounter with the ion pore of ShBΔ6−46.

Ionic Strength Dependence of k_{on}^{in} and k_{off}^{in}. We now use eq 18 and the interaction potential of eq 19 to predict the ionic strength of k_{on}^{in}. For simplicity we assume that the ligand is tethered to the center of the receptor sphere. Then $d_0 = a$ and

$$k_{on}^{in} = k_{on}^{bi}p(a)/\int_a^\infty 4\pi r^2\, p(r)\exp[-\beta U(r)]dr \qquad (21)$$

k_{on}^{in} is predicted by eq 21 to have a much weaker dependence on ionic strength than k_{on}^{bi}, decreasing 4-fold when the ionic strength is increased from 50 to 600 mM. Figure 4 shows that such a weaker dependence is in quantitative agreement with the experimental result of Murrell−Lagnado and Aldrich.[20, 21]

The intramolecular unbinding rate constant was observed to be nearly the same as the bimolecular counterpart and appeared to be a constant when the ionic strength was changed.[20] This is entirely consistent with eq 17.

Linker Length Dependence of k_{on}^{in}. Hoshi et al.[22] observed that the intramolecular binding rate is increased when the linker length is reduced by deletion. Conversely, a decrease in binding rate was observed when the linker length was extended by insertion. Specifically, when 15 residues were deleted, the binding rate increased by 3-fold, and when 41 residues were inserted, the rate decreased by 19%. This trend is qualitatively predicted by eq 21. Assuming $L = 100$ for wild-type ShB, eq 21 predicts that k_{on}^{in} increases by 13% when $L = 85$ and decreases by 26% when $L = 141$.

Discussion

We have developed a theory relating the kinetics of the intramolecular binding of a ligand tethered to a receptor and the kinetics of the bimolecular binding of the ligand to the receptor. The theory accounts for both the long-range interactions between the ligand and the receptor and the effect of the linker [via the probability density $p(r)$ of the end-to-end vector]. Its application to the binding of the inactivation N-terminal to the ion pore of the *Shaker* potassium channel leads to the rationalization of a number of important experimental observations. (1) The weaker ionic strength dependence of the intramolecular binding rate constant k_{on}^{in} is quantitatively reproduced by the relation between k_{on}^{in} and k_{on}^{bi}. (2) The linker length (L) dependence of k_{on}^{in} is qualitatively predicted by the L depen-

dence of $p(r)$. (3) The fact that $k_{off}^{in} = k_{off}^{bi}$ and both are insensitive to the change in ionic strength is due to the stereospecificity of the binding site.

Szabo et al.[23] have calculated the rate of end-to-end contact formation for a Gaussian chain based on the mean first passage time. This theory has been used to analyze experimental data on the rate of intrachain contact formation in cytochrome *c*.[24] Our theory may be considered as a generalization by the introduction of long-range interactions between the ligand and the receptor. We also derive results for stereospecific binding in addition to those for the binding of a spherical ligand to a spherical receptor.

Timpe and Peller[25] have attempted to explain the linker length dependence of the binding rate constant observed by Hoshi et al.[22] The formula used by them, $k_{on}^{in\prime}/k_{on}^{in} = (1 + \Delta L/L)^{-3/2}$, is equivalent to eq 18b (i.e., without consideration of long-range electrostatic interactions) with $p(d_0)$ given by the Jacobson-Stockmayer result. This formula predicts a 28% increase in k_{on}^{in} for $\Delta L = -15$ and a 40% decrease in k_{on}^{in} for $\Delta L = 41$. The introduction of long-range electrostatic interactions in our theory for the inactivation kinetics is important, since experiments have shown that the binding of the N-terminal to the channel pore is strongly affected by these interactions.[18,20]

The rapid inactivation of ion channels is crucial since termination of one action potential must occur before the arrival of the next. It is instructive to compare the termination mechanism via ion pore occlusion for the propagation of action potentials along the axon of a neuron to the termination mechanism for the propagation of action potentials across cholinergic synapses. That propagation occurs through the binding of acetylcholine (Ach) to its receptor, which triggers the opening of the receptor as an ion channel. An ACh molecule released from the receptor must be degraded on a millisecond time scale (before the next action potential arrives) so that it does not have a second chance of binding the receptor. The task of degradation falls on acetylcholinesterase (AChE), which binds ACh with a rate constant of 5×10^8 $M^{-1}s^{-1}$.[26] Such a high rate constant is a result of electrostatic enhancement.[27] A millisecond clearance time means that the AChE concentration must be at least 2 μM. Indeed there are 2600 catalytic subunits of AChE tethered to each μm^2 of basal lamina in the synapse,[28] which corresponds to a concentration of 20 μM. The tethering is critical because otherwise the AChE concentration may diminish as a result of diffusion. In passing, we note that, in the absence of electrostatic interactions, the binding rate constant of ACh to AChE is only 7.4×10^6 $M^{-1}s^{-1}$,[27] which would result in a clearance time of 7 ms. This excessively long time underscores the importance of the electrostatic rate enhancement.

If the binding of the activation N-terminal of ShB were to occur in a bimolecular fashion, a rate constant of 1.5×10^5 $M^{-1}s^{-1}$ in the absence of electrostatic interactions would require that the N-terminal peptide be maintained at a concentration of 7 mM. Presence of the electrostatic interactions increases the rate constant to 5×10^6 $M^{-1}s^{-1}$ (at $I = 150$ mM) and lowers the required concentration of the N-terminal peptide to 0.2 mM. The difficult task of maintaining such a high concentration is simply solved by the covalent linking of the N-terminal to the ion channel.

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