A Random Flight Chain Model for the Tether of the *Shaker* K^+ Channel Inactivation Domain

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ABSTRACT Rapid inactivation of *Shaker* K^+ channels occurs when a domain in the amino terminal region of the channel protein blocks the pore. Some part of the sequence between the inactivating domain and the first transmembrane segment may form a flexible tether. We consider the possibility that the tether has no secondary structure, but is rather a polypeptide random coil. The local concentration of the tethered inactivation domain and the dependence of the inactivation rate on chain length can then be calculated by using the Jacobson-Stockmayer equation. A chain of 30–100 amino acids is consistent with the sensitivity of the inactivation rate to chain length mutations.

INTRODUCTION

In *Shaker* K^+ channels the core of the protein, which comprises the pore and voltage sensor, is attached to an amino terminal inactivating domain by a link of unknown secondary structure (Hoshi et al., 1990; Zagotta et al., 1990; Murrell-Lagnado and Aldrich, 1993a,b). Inactivation occurs when the cytoplasmic amino terminal domain occludes the open pore (Demo and Yellen, 1991). Shortening the polypeptide chain between the inactivation domain and the first transmembrane region accelerates the rate of inactivation, whereas lengthening the chain slows the rate. In a simple interpretation of these results the chain length mutations do not alter the chemistry of binding, but rather change the local concentration of the blocking particle in the region of its binding site. We consider the consequences of assuming that some part of the protein between the amino terminal blocking domain and the core has no secondary structure, but is instead a random flight coil. This hypothesis leads to the prediction that the inactivation rate will be proportional to the $-3/2$ power of the chain length for long chains, and allows the local concentration of the inactivating domain in the neighborhood of its binding site to be calculated and compared with the experimental value.

The evidence for a "ball and chain" inactivation mechanism is due to Hoshi et al. (1990) and Zagotta et al. (1990). In their experimental system, in which the *Shaker* B K^+ channel splice variant (Schwarz et al., 1988) is expressed in *Xenopus* oocytes, the channel is thought to be a homotetramer (MacKinnon, 1991). Each channel therefore has four inactivation domains, which block independently (MacKinnon et al., 1993). Experiments supporting the ball and chain mechanism for inactivation include a deletion analysis, which mapped the inactivation domain to roughly 20 amino acids at the amino end of the protein (Hoshi et al., 1990), and the demonstration that a free 20 amino acid peptide with sequence derived from the inactivating domain can block non-inactivating mutant channels (Zagotta et al., 1990). Fig. 1 illustrates the situations for the tethered inactivation domain and for the free peptide.

RESULTS AND DISCUSSION

Experimental determination of the local concentration

Comparing the rate constants for the tethered and untethered arrangements allows the local concentration of the inactivation domain to be measured. In the tethered case the rate constant for entering the inactivated state from the open state, k_1 (s^{-1}), is first order. When the blocking peptide is free, the rate constant k_2 ($M^{-1}\ s^{-1}$) is second order. The ratio of these two rate constants defines a local concentration (c_L) of the ball in the neighborhood of its binding site.

$$c_L = k_1/k_2 \quad (1)$$

With the units indicated above, c_L has the dimensions of molarity. It can be viewed as the concentration of free peptide that matches the blocking rate of the tethered inactivation domain.

Equilibrium constants for the unimolecular and bimolecular processes can likewise be combined to yield a value for c_L. It will be the same as that defined by Eq. 1, provided that the reverse rate constants, for leaving the inactivated state, are identical for the tethered and untethered cases. Murrell-Lagnado and Aldrich (1993a) have defined the local concentration in this way.

These definitions have their simplest interpretations when the chemistry of the binding reaction is identical for the free peptide and the tethered inactivation domain. The tether then serves to diminish the entropy loss on bringing two species together. There are a number of examples of other reactions where such benefits of a prior attachment on the ultimate binding step occur. Multivalent antigen-antibody

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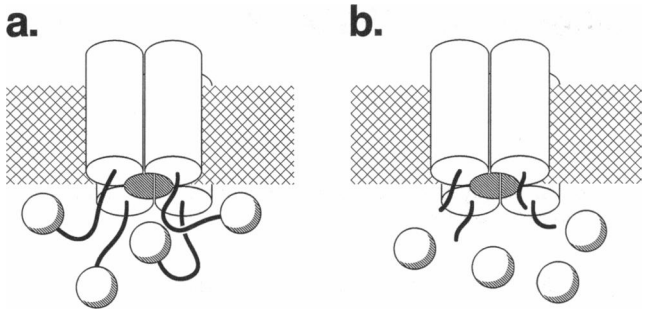

FIGURE 1 An illustration of homotetrameric *Shaker* B channels with the inactivation domains (*balls*) intact (*a*), or with inactivation domains deleted but blocking peptide present (*b*).

associations (Crothers and Metzger, 1972), the two-headed myosin molecule associating with fibrous actin, and numerous substrate enzyme reactions may be viewed in this way (Jencks, 1981).

Equation 1 can be used to estimate the local concentration from published data. From studies in physiological saline Hoshi et al. (1990) found a value of about 625 s^{-1} for the rate constant of channel inactivation. As inactivation can be mediated by one of four independent inactivating domains, we take $k_1 = 156\ s^{-1}$. Murrell-Lagnado and Aldrich (1993a) report a companion value of $k_2 = 4.7 \times 10^6\ M^{-1}\ s^{-1}$, which gives $c_L = 33\ \mu M$ from Eq. 1. Murrell-Lagnado and Aldrich (1993b) have also shown that increasing the ionic strength of the medium diminishes the rate constants for channel inactivation and peptide block, indicating that there are electrostatic interactions between the inactivating domain or peptide and its binding site. Furthermore, there is a differential effect of ionic strength on the magnitudes of the bimolecular and unimolecular rate constants, with the bimolecular rate constant being more sensitive to the salt concentration. Consequently, the local concentration, as defined by Eq. 1, rises with ionic strength to about 75 μM measured in 600 mM *N*-methylglucamine. Seventy-five μM may be a more useful value for our purposes, as electrostatic effects would be reduced or absent.

Murrell-Lagnado and Aldrich (1993b) proposed several mechanisms to account for the differential sensitivity of the rate constants to ionic strength. An additional possibility is that the chain itself carries a net charge, which would cause it to adopt a more extended conformation than would a similar, uncharged chain. Increasing salt concentration would then shield these charges, allow the tether to become more compact, and increase the local concentration of the inactivation domain. This effect could make the unimolecular rate constant less sensitive to inceasing salt than the bimolecular constant, as observed. If the wild-type tether behaves as a polyelectrolyte, this behavior would be accentuated by increasing the net charge of the tether. The expanded volume should be a function of the square of the net charge density, as the electrostatic effect is the same whether the net charge is positive or negative. Increasing the net charge should lead to a much lower local concentration of the inactivation domain and to much slower inactivation. The relationship between electric charge and volume in polymers has been the subject of much theoretical work (see Fixman and Skolnick, 1978).

Theoretical treatment of the local concentration

Approximately 200 amino acids separate the amino terminal blocking domain from the first transmembrane segment of *Shaker* B. Of these 200 amino acids roughly 100, on the carboxyl side of the sequence, are important for subunit assembly and may not contribute to the tether (Li et al., 1992). Some portion of the remaining sequence forms the tether. Assuming that the tether has no secondary structure, we may describe it as random flight coil. This means that the backbone of the polypeptide at any instant approximates a three-dimensional random walk. The density function for the probability that one end of a polymer is located in a volume element $(dx)(dy)(dz)$ at a distance r from the other end is given for long chains by

$$W(r) = 2\left(\frac{3}{2\pi\langle r^2\rangle}\right)^{3/2} \exp\left(-\frac{3\,r^2}{2\langle r^2\rangle}\right) \qquad (2)$$

where $r^2 = x^2 + y^2 + z^2$ and $\langle r^2\rangle$ is the mean square end-to-end distance (Flory, 1969). The origin of coordinates is taken as the point of emergence of the tether from an ordered region of the protein, with the z coordinate perpendicular to the membrane surface. A factor of 2 appears because the volume accessible to the tether is confined to one side of the membrane. $W(r)$ is normalized if x and y range from $-\infty$ to ∞, and z from 0 to ∞.

The mean end-to-end distance, $\langle r^2\rangle$, can be written

$$\langle r^2\rangle = \alpha^2\langle r^2\rangle_0 \qquad (3)$$

where $\langle r^2\rangle_0$ is the mean square end-to-end distance of a chain unperturbed by excluded volume effects or by the electrostatic repulsions present in a polyelectrolyte. The latter effects would both tend to increase the end-to-end distance compared to the unperturbed chain and are taken into account by the dimensionless factor α (Flory, 1953).

If the binding site of the inactivation domain in the pore is near the origin of the random coil, or $r \ll \langle r^2\rangle^{1/2}$, then the exponential term in Eq. 2 is approximately 1 and the local concentration of the inactivation domain can be identified with the pre-exponential factor:

$$j = 2\left(\frac{3}{2\pi\langle r^2\rangle}\right)^{3/2} \qquad (4)$$

in molecules/$Å^3$. Omitting the factor of 2, Eq. 4 was introduced by Jacobson and Stockmayer (1950) in their treatment of the cyclization occurring in condensation polymerization. Wang and Davidson (1966) used a similar approach in the study of competitive cyclization versus linear growth by lambda phage DNA.

Unperturbed polypeptide dimensions

The mean square end-to-end distances for several synthetic homopolypeptides have been determined and compared with theoretical predictions (Brant and Flory, 1965). In solvents in which excluded volume effects and electrostatic interactions are minimal, i.e., $\alpha = 1$,

$$\langle r^2 \rangle = \langle r^2 \rangle_0 = C N l^2 \quad (5)$$

where N is the number of amino acid residues, l is the distance between α carbons (3.8 Å), and C is the characteristic ratio. The characteristic ratio is related to the stiffness of a chain. For a freely jointed chain, $C = 1$. In polypeptides, however, there is some correlation between the directions of neighboring segments due to the tetrahedral orientations of the α carbon bonds and to restrictions on rotation imposed by the various side chains. The characteristic ratio is a function of N, the number of segments in a chain; as N increases C rises to a limiting value that depends on the nature of the monomer. For the charged polypeptides poly(L-glutamate) and poly(L-lysine) C is about 9 at high ionic strength. A similar value of C was calculated from earlier data for poly(benzyl-L-glutamate) (Brant and Flory, 1965). The characteristic ratio for polyglycine has been calculated to be 2; the low value is due to the lack of a side chain (Flory, 1969). Polymers of proline, in which rotation around the C_α-N bond is impeded, are calculated to have $C = 116$ and are virtually helical, save at very high molecular weights.

Combining Eqs. 4 and 5 and converting to mol/L gives:

$$c_L = 10^{27} N_A j = 20\,(CN)^{-3/2} \quad (6)$$

where N_A is Avogadro's number.

Fig. 2 *A* shows c_L as a function of N and three values of C. The local concentration of one end of a random flight polypeptide with respect to the other end falls as the number of segments increases. The flexibility of the poly(glycine) chain ($C = 2$) allows it to adopt a large number of tortuous configurations in which the ends are near each other, giving the greatest local concentration for a given N.

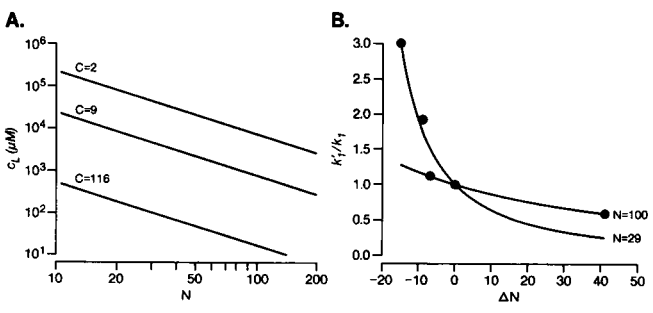

FIGURE 2 The effects of characteristic ratio, chain length, and chain length mutations on the local concentration. (*A*) A log-log plot of the local concentration as a function of chain length from Eq. 6, with the characteristic ratios for poly(glycine) (2), poly(alanine) (9), and poly(proline) (116). (*B*) The rate constants associated with three chain length deletions and one insertion (Hoshi et al., 1990) were normalized to the wild-type value and plotted as a function of mutation size (*circles*). Equation 7 was fit to the results, with the best fit to all points occurring with $N = 29$.

The hypothesis that the tether of the *Shaker* K^+ channel behaves as a random flight coil leads to the prediction that the local concentration of the inactivation domain, and hence the inactivation rate constant, will vary with the $-3/2$ power of the chain length. This analysis separates the effect of chain length on the inactivation rate from the chemistry of binding by the inactivation domain, which is determined empirically through the bimolecular rate constant. The $-3/2$ power dependence of inactivation rate on chain length is different from that proposed by Liebovitch et al. (1992). Using a random walk simulation, those authors determined the form of the probability density function for the waiting time for the inactivation domain to make its first visit to the binding site. This approach treats inactivation as a variety of diffusion problem. As Zagotta et al. (1990) point out, however, the rate constant of block by the free peptide is significantly lower than expected for a diffusion-limited reaction. Our approach makes no commitment to a diffusion-limited rate.

For a chain of 100 segments and a characteristc ratio of 9, $c_L = 740$ μM. This local concentration is considerably higher than the 75 μM measured for the *Shaker* K^+ channel inactivation domain. Increasing either N or C would reduce the calculated value of c_L. We expect, however, that the number of segments in a random flight coil is not likely to be greater than 100. As the length of a polypeptide random coil increases, the probability that it will acquire secondary structure increases. Because there are small enzymes of about 100 amino acids, it seems unlikely that N is much larger.

The difference between the calculated and observed local concentrations may be due to uncertainty in choosing a suitable characteristic ratio or to excluded volume effects. Considering first the characteristic ratio, it seems that a chain much stiffer than poly(glutamate) or poly(lysine) is required to give a local concentration of 75 μM. Values of C for polypeptide chains composed of mixed amino acids, as opposed to homopolymers with charged side chains, have not been determined experimentally, and perhaps they differ significantly from the homopolymers tested. Alternatively, the tether, or part of it, may have a secondary structure that contributes to the overall stiffness of the chain.

The measurements for homopolymeric peptides that have been made were obtained in conditions that minimize excluded volume and electrostatic effects (Brant and Flory, 1965). In the cytoplasm of a cell, a chain may adopt a more extended average conformation than it would in an ideal solvent, resulting in a lower local concentration. This effect could be included in the calculation of the local concentration by setting $\alpha > 1$ in Eq. 3, but we have no independent experimental evidence for an appropriate choice for α.

Does the assumption that $r \ll \langle r^2 \rangle^{1/2}$ hold? Setting $c_L = 75$ or 740 μM and using Eqs. 5 and 6 gives $\langle r^2 \rangle^{1/2} = 245$ or 114 Å, respectively. The distance from the origin of the tether to the binding site of the inactivation domain is not

known but is likely to be similar to the diameter of one or two α helices, or 4 to 8 Å. Thus the analogy to a cyclization reaction is plausible.

An estimate of chain length

The effects of changes in chain length on the inactivation rate constant, as reported by Hoshi et al. (1990), can be used to estimate the number of segments in the chain. From Eqs. 1 and 4 we can write

$$\frac{k_1'}{k_1} = \left(1 + \frac{\Delta N}{N}\right)^{-3/2} \tag{7}$$

where k_1' is the rate constant associated with a chain length mutation. Delta N is the number of amino acids inserted or deleted and is positive for insertions. Equation 7 asssumes that $k_2' = k_2$, i.e., that the mutations do not alter the function of the inactivation domain. The equation also assumes that the change in length of the chain has not altered its characteristic ratio, for example, by changing the glycine or proline content. Likewise α, which accounts for the electrostatic or excluded volume effects, is taken to be the same for the mutant and wild-type chains.

Hoshi et al. (1990) have measured the effects of three deletions and one insertion on the rate constant k_1 for *Shaker* B. In Fig. 2 *B* we have taken data from their Fig. 3 *C*, normalized to k_1 for the wild-type channel, and plotted it as a function of delta N. The rate constant k_1 declines when the length of the chain increases, as noted by Hoshi et al. (1990). The line corresponding to the ratio of rate constants calculated from Eq. 7 with $N = 29$ is the least-squares best fit to the data. This result indicates that a random flight coil of about 30 amino acids is compatible with the chain length mutation experiments. This procedure may not give a very accurate estimate of N, however. Although the characteristic ratio has a limiting value for long chains, for short chains it varies with chain length and perhaps should not be treated as a constant in deriving Eq. 7. Experimental results with polydimethylsiloxane confirm that the expected dependence of the local concentration on the $-3/2$ power of the chain length does not hold for small N (Flory, 1969). The curve calculated for $N = 100$ fits the points for the 41 amino acid insertion and the smallest deletion, 7 amino acids (Fig. 2 *B*). Thus 100 amino acids is perhaps a better estimate for N. These estimates for N are consistent with the number of amino acids available for the chain, as determined from the primary structure. This agreement contrasts with the estimate of the local concentration, which is tenfold too high. The greater usefulness of Eq. 7 is probably due to the fact that C and α, which are poorly known, disappear when the data are viewed as the ratio of the rate constants.

In summary, the simplest assumption about the structure of the tether, that it has no secondary structure, provides a way to calculate the dependence of the inactivation rate on the length of the tether and the local concentration of the inactivating domain in the neighborhood of its binding site. The random flight coil model predicts that the inactivation rate constant will vary with the $-3/2$ power of the chain length. Three parameters required for the calculation of the local concentration, the number of segments in the chain, the characteristic ratio of the chain, and the extent of excluded volume effects, are not known accurately. Using reasonable estimates for the number of segments and the characteristic ratio, the calculated local concentration is 10 times greater than the measured one. The discrepancy may be due to excluded volume effects, which would lower the local concentration, but which are difficult to quantitate. It is nevertheless possible to estimate the number of segments of the *Shaker* channel tether from the effects of chain length mutations on the inactivation rate; this estimate, that the tether comprises 30–100 amino acids, is not sensitive to the characteristic ratio or to excluded volume effects.

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