Diffusion-Controlled Binding of a Peptide Neurotoxin to Its K^+ Channel Receptor†

Christopher Miller

Howard Hughes Medical Institute, Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02254

Received December 13, 1989; Revised Manuscript Received February 27, 1990

ABSTRACT: Single Ca^{2+}-activated K^+ channels were reconstituted into planar lipid bilayer membranes, and the effect of charybdotoxin, a pore-blocking peptide from scorpion venom, was studied. In particular, the effect of solution viscosity on the kinetics of block was assessed in order to test the idea that toxin binding is diffusion-controlled. This idea is supported by the strictly inverse relation between solution viscosity and the rate constants of both association and dissociation of peptide with the K^+ channel mouth. However, at an ionic strength high enough to suppress local electrostatic potentials, the diffusion-controlled on-rate constant is surprisingly low, 10^5 M^{-1} s^{-1}. These slow, viscosity-dependent kinetics may be understood if charybdotoxin can attain the bound state only from a rare set of encounters with the K^+ channel.

Charybdotoxin (CTX) is a scorpion venom derived peptide which blocks several types of K^+-specific ion channels at nanomolar concentrations (Miller et al., 1985; Hermann & Erxleben, 1987; MacKinnon et al., 1988; Sands et al., 1989; Schweitz et al., 1989; Grinstein & Smith, 1989). A series of detailed mechanistic experiments has shown that the toxin inhibits one of these, the high-conductance Ca^{2+}-activated K^+ channel, by binding to and physically plugging the externally facing "mouth" of the K^+ conduction pore (Anderson et al., 1988; MacKinnon & Miller, 1988; Miller, 1988). Moreover, it is known that a strong electrostatic interaction favors CTX binding; the channel mouth carries carboxylate residues which attract the positively charged toxin (Anderson et al., 1988; MacKinnon & Miller, 1988, 1989; MacKinnon et al., 1989).

This study is directed toward developing a picture of the molecular recognition of CTX by its receptor on the Ca^{2+}-activated K^+ channel. The channel binds CTX with high specificity. For example, toxin block is greatly weakened by mild treatments of CTX, such as lysine acylation (Smith et al., 1986), tyrosine monoiodination (Lucchesi et al., 1989), or removal of the two N-terminal amino acids by chymotrypsin (Smith, 1988); furthermore, related venom peptides with impressive sequence homology to CTX, such as noxiustoxin (Valdivia et al., 1988), fail to block this K^+ channel. In spite of this specificity in peptide–receptor recognition, the association rate of CTX with the Ca^{2+}-activated K^+ channel is quite high, on the order of 10^7 M^{-1} s^{-1} at physiological ionic strength (Anderson et al., 1988; MacKinnon et al., 1989). This high value suggests that diffusion of the peptide up to the channel might be the rate-determining step in the binding reaction. This high rate raises the possibility that special "steering" mechanisms may guide the toxin toward the channel mouth, and perhaps orient it as it approaches the receptor site, as has been proposed for several fast enzymatic reactions (Getzoff et al., 1983; Sharp et al., 1987; Northrup et al., 1988; Ravindran & Moczydlowski, 1989).

These experiments aim to test whether the rate-determining step for CTX binding is, in fact, diffusion of toxin up to the channel. It is well understood that a high value for a second-order rate constant, while suggestive that a reaction may be diffusion-limited, is not a rigorous criterion to conclude that this condition holds (Schurr, 1970a,b). A direct test of this idea can be made by varying the diffusion coefficient of the toxin by increasing the viscosity of the aqueous medium, and observing the effect on reaction rate, as has been done with numerous enzyme-catalyzed reactions (Brouwer & Kirsch, 1982; Lim et al., 1988; Guha et al., 1988; Gates & Northrop, 1988). The rationale of this approach is based on the Stokes–Einstein relation, which states that the diffusion coefficient (D) of a solute of effective radius r varies inversely with the medium viscosity (η):

$$D = kT/6\pi r\eta \quad (1)$$

where k is the Boltzmann constant and T is temperature. Accordingly, if CTX binding is diffusion-controlled, then *both* association and dissociation rate constants must vary inversely with solvent viscosity (Schurr, 1970a). The results here demonstrate that CTX binding is diffusion-controlled. Moreover, an examination of the effects of ionic strength on the CTX association rate shows that local electrostatic effects at the external surface of the K^+ channel contribute to a high rate of CTX binding.

Materials and Methods

Biochemical Methods. Previously described procedures were used for the preparation of plasma membrane vesicles from rat skeletal muscle (Moczydlowski & Latorre, 1983) and CTX from *Leiurus quinquestriatus* venom (Smith et al., 1986). Lipids used for forming planar bilayer membranes were obtained from Avanti Polar Lipids, Inc. Potassium chloride with negligible contamination by Ba^{2+}, required for single-channel measurements, was "puratronic" grade from Alpha Products, Danvers, MA.

Single-Channel Measurements. Membrane vesicles containing single Ca^{2+}-activated K^+ channels were fused into planar lipid bilayers formed from decane solutions of 85% 1-palmitoyl-2-oleylphosphatidylethanolamine/15% 1-palmitoyl-2-oleylphosphatidylcholine, as described (Anderson et al., 1988). Incorporation of channels was accomplished by the standard osmotic fusion method (Miller & White, 1980); channels were inserted in the presence of a large osmotic gradient, with 150 mM KCl/10 mM HEPES/5 mM KOH/20 μM $CaCl_2$/0.2 mM $MgCl_2$, pH 7.4, in the "internal" chamber and 10 mM HEPES/5 mM KOH/0.1 mM EGTA, pH 7.4, in the "external" chamber. After the appearance of a single channel, KCl was added to the external chamber to a final concentration of 150 mM to equalize the K^+ concentrations

† Supported by NIH Grant GM-31768.

0006-2960/90/0429-5320$02.50/0

Table I: Insensitivity of Channel Gating to External Sucrose[a]

	τ_1 (ms)	A_1	τ_2 (ms)	A_2	$\langle\tau\rangle$ (ms)	P_o
control	**1.8** (1.9)	**0.17** (0.79)	**11** (15)	**0.83** (0.21)	**10.6** (6.2)	0.63
sucrose	**1.8** (1.8)	**0.25** (0.63)	**10** (10)	**0.75** (0.37)	**9.8** (5.6)	0.64

[a] A single channel was incorporated into a planar bilayer, as in Figure 1, and the indicated parameters of channel gating were determined, in the presence and absence of 1 *m* sucrose in the external medium. Open and closed durations were well fit to double-exponential probability distributions, and the fast and slow time constants, τ_1 and τ_2, as well as the amplitudes of these relaxations, A_1 and A_2, are reported. Quantities in parentheses refer to closed times, and boldface quantities refer to open times. In addition, the overall mean open and closed times, $\langle\tau\rangle$, and the open probabilities, p_o, are reported. The total number of events to construct distributions was 2930. The holding potential was 35 mV.

on the two sides of the bilayer and to suppress further channel incorporation. Single-channel records were analyzed by a data acquisition computer running an on-line "probability-clamp" program, so that the open probability could be adjusted, by setting the applied voltage, to be in the range of 0.6–0.8 (Anderson et al., 1988). Under these conditions, the applied voltage was usually in the range 35–45 mV, with the external solution defined as zero voltage. Temperature was 22 ± 1 °C in all experiments.

CTX blocking kinetics were measured by collecting 100–600 discrete transitions of the single channel into and out of the CTX-induced blocked state, and calculating association and dissociation rate constants from the exponential distributions of dwell times of blocked and unblocked states (Anderson et al., 1988). To induce block, CTX (20–65 nM) was added to the external chamber, along with 50 μg/mL bovine serum albumin to prevent loss of CTX to the chamber walls, and after 2–3 min of vigorous stirring, the single channel was recorded for up to 2 h. To change solutions during an experimental run, the bilayer chambers were extensively perfused with fresh medium. Since high concentrations of nonelectrolytes were used to change viscosity, solute concentrations here are reported in units of millimolal (m*m*), rather than millimolar.

Results

This study is aimed at assessing the effects of solution viscosity on CTX block of single Ca^{2+}-activated K^+ channels. However, before embarking on the central experiments, we must be assured that the extreme conditions used to alter viscosity, i.e., very high concentrations of nonelectrolytes in the medium, do not adversely affect the behavior of the channel. Figure 1 illustrates qualitatively that inclusion of 1 *m* sucrose in the external medium does not affect the kinetics of channel gating, a process which is highly sensitive to numerous external factors, such as voltage, Ca^{2+} and Mg^{2+} concentration, and solution ionic strength (Golowasch et al., 1986; Moczydlowski & Latorre, 1983; Moczydlowski et al., 1985; Barrett et al., 1982; MacKinnon et al., 1989). In the figure, the gating of single Ca^{2+}-activated K^+ channels reconstituted into planar phospholipid bilayers (Anderson et al., 1988) is shown in symmetrical 150 mM K^+, with and without sucrose present in the external solution. Channel opening and closing kinetics are similar under the two conditions, and the single-channel currents are virtually identical. Table I makes this point quantitatively by reporting the parameters for the double-exponential distributions of dwell times of the open and closed states of the channel; probability distributions of these open and closed durations, which are sensitive manifestations of channel gating, are unaffected by sucrose. It is safe to conclude, therefore, that the addition of high sucrose concentrations to the external solution does not induce gross misbehavior in the channel protein; indeed, it does not noticeably alter the channel's open–closed equilibrium, its Ca^{2+} dependence, or its variation with applied voltage (data not shown). Furthermore, 1 *m* sucrose does not affect the activity coefficient of K^+, since the reversal potential of the open-

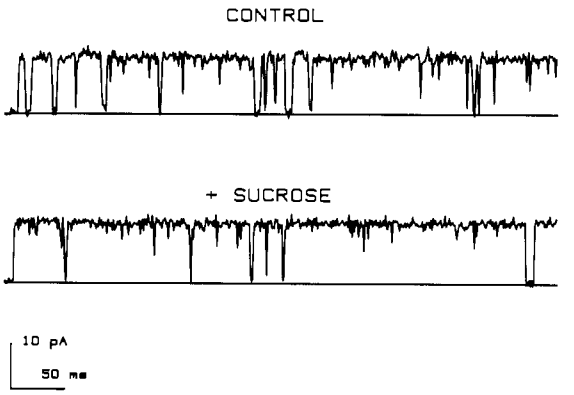


FIGURE 1: Effect of high sucrose concentration on Ca^{2+}-activated K^+ channel gating. A single Ca^{2+}-activated K^+ channel was incorporated into a planar bilayer, and steady-state records were collected at a holding potential of 35 mV. Raw single-channel records are shown, under control conditions and after perfusion of the external chamber with a solution containing 1 *m* sucrose. Channel openings in this and subsequent figures are always upward; zero-current level is indicated by the horizontal line. In this experiment, the open probability was 0.85 for the control and 0.90 with sucrose present.

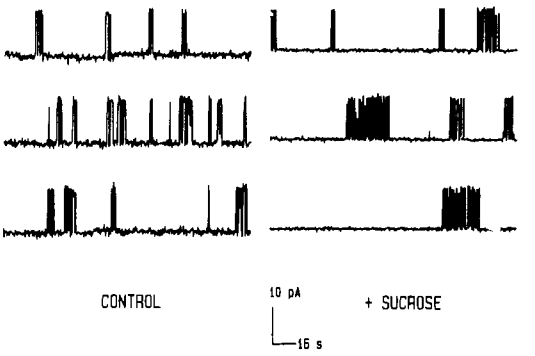


FIGURE 2: Sucrose slows CTX block kinetics. Single-channel records in the presence of 50 n*m* CTX are shown for a channel under control conditions, and then after inclusion of 0.7 *m* sucrose in the external solution. Each trace shows a continuous 8-min record. The holding voltage was 37 mV.

channel current–voltage relation was always within 2 mV of zero, with or without sucrose present.

We are now in a position to examine the effect of external solution viscosity on CTX block of this K^+ channel. Figure 2 illustrates the random fluctuations of single Ca^{2+}-activated K^+ channels induced by addition of CTX to the external solution. These single-channel recordings, which are displayed on a much slower time scale than in Figure 1, reveal the binding of *individual* CTX molecules, and thus contain information about the association and dissociation steps. Each long-lived nonconducting interval begins with the binding of a single CTX molecule to the single channel, and ends when that particular toxin molecule dissociates (Anderson et al., 1988; Miller et al., 1985); accumulation of many such fluctuations, under equilibrium conditions, allows the measurement of the toxin on- and off-rate constants, k_{on} and k_{off}, from the

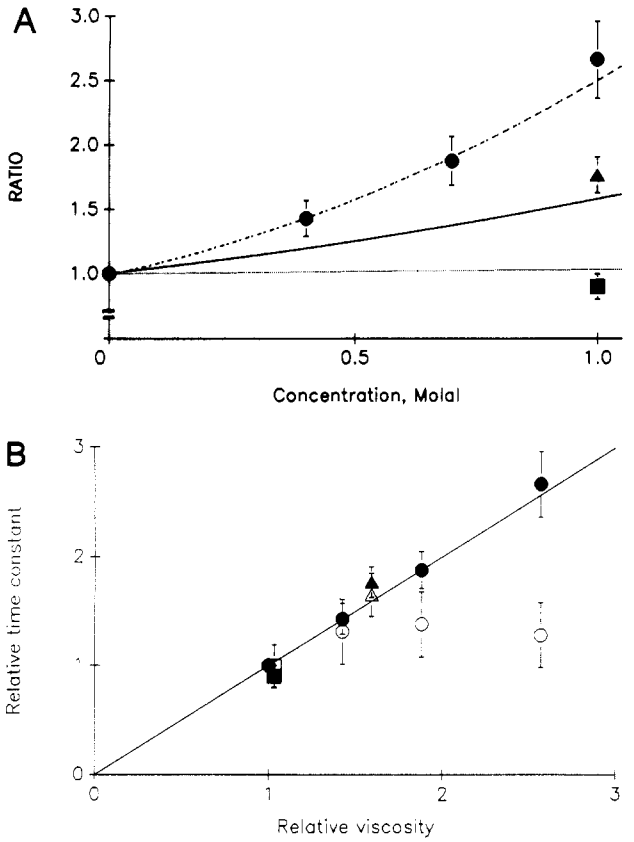


FIGURE 3: Effect of solution viscosity on CTX block kinetics. CTX association and dissociation kinetics were measured on single Ca^{2+}-activated K^+ channels under control conditions, and with various viscogenic agents included in the external solution. Each point represents the mean ± SE of three to eight separate determinations, each on a different channel, using CTX concentrations in the range 35–50 nM. Values of on- and off-rate constants were compiled from these determinations, and were normalized to the value measured in solution with no added viscogenic agent. Absolute values of rate constants with no added viscogenic agent were $k_{on} = 4.6 \times 10^6$ M^{-1} s^{-1} and $k_{off} = 0.069 \pm 0.003$ s^{-1}. (A) Effect of urea (squares), sucrose (circles), and glucose (triangle) on CTX block time. Average block times were determined and reported as the ratio of this value to that with no added viscogenic agent. Curves show the corresponding ratios of solution viscosity normalized to that of 0.15 M KCl at 20 °C, and were drawn from tabulated values (Weast, 1983). (B) Correlation between blocking kinetics and relative viscosity. Relative mean block times (closed symbols) and unblock times (open symbols) are plotted vs relative viscosity. The solid line has slope of unity.

measured average dwell times of the blocked and unblocked channel, $\langle \tau_b \rangle$ and $\langle \tau_u \rangle$, and the CTX concentration (Anderson et al., 1988):

$$k_{on} = 1/\langle \tau_u \rangle [CTX] \quad (2)$$

$$k_{off} = 1/\langle \tau_b \rangle \quad (3)$$

Mere visual comparison of the raw channel traces of Figure 2 shows that adding 0.7 *m* sucrose to the external medium, a maneuver that increases the viscosity by a factor of 1.9 (Weast, 1983), slows down the toxin association–dissociation kinetics. In the higher viscosity medium, both blocked and unblocked states are, on average, longer lived than in sucrose-free control solution. Is this effect of sucrose a consequence of the increased solution viscosity or of other unspecified effects due to the high sucrose concentration in the external solution? Figure 3A argues strongly that the toxin kinetics respond directly to solution viscosity; here, the relative block times (data points) and solution viscosities (continuous curves) show striking quantitative agreement for three nonelectrolytes of varying ability to alter solution viscosity: sucrose, glucose, and urea. This experiment focuses upon the mean block time, since this measures the reciprocal of the dissociation rate constant (eq 3), which is a first-order rate constant, and hence is uncomplicated by changes in CTX activity coefficients induced by high concentrations of nonelectrolytes (see below).

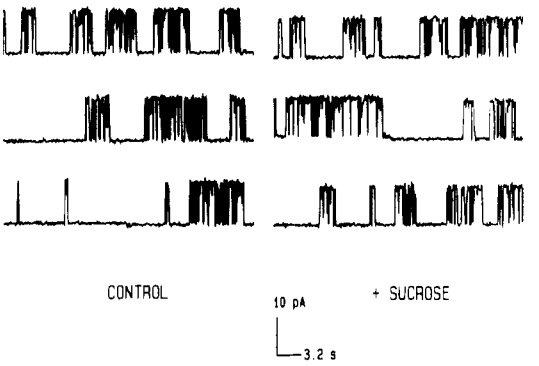


FIGURE 4: Lack of viscosity effect on block by Ba^{2+}. Traces were recorded for single channels in the presence of 4 mM external Ba^{2+} at 10 mV, conditions under which blocking events can be observed for individual Ba^{2+} ions. In this experiment, under control conditions, mean block times and unblock times were 4.6 and 2.6 s, respectively ($N = 92$); in the presence of 1 *m* sucrose, mean block times and unblock times were 4.3 and 2.9 s ($N = 83$).

Further support for diffusion control of the CTX binding reaction is reported in Figure 3B. Both block times and unblock times (corresponding to reciprocal off- and on-rate constants) are tightly correlated with solution viscosity, over a range of concentrations of different viscogenic agents. As above, the relative block time (closed points) is quantitatively identical with the relative viscosity up to at least a 2.6-fold viscosity increase (1 *m* sucrose). Likewise, the unblock times (open points) are well-behaved up to relative viscosity of 1.6, but above this value, they depart from the line of equality with increasing severity. This departure is not surprising, since it is known that high concentrations of polyhydric solutes can anomalously increase the activity coefficients (and hence "effective concentrations") of proteins in aqueous solution (Lee & Timasheff, 1981); indeed, high sucrose concentrations cause the toxin to bind more tightly to the channel, as though the toxin activity is being raised, in analogy with the effects of sugars and polyols on tubulin and ribonuclease (Pittz & Timasheff, 1978).

Because of the problems associated with adding large concentrations of sugars to solution, it would be desirable to have a control experiment with a "poor" blocker, i.e., an open-channel blocker with a *slow* association rate, far below the diffusion limit. Barium ion provides an opportunity to perform such an experiment. This ion is known to enter the open channel and produce long-lived blocking events (Vergara & Latorre, 1983; Benham et al., 1985; Miller et al., 1987), but with an association rate constant below 100 M^{-1} s^{-1}, about 5 orders of magnitude less than for CTX. Ba^{2+} binding is so slow because the ion must cross large energy barriers to attain its site, which is located deeply within the channel's conduction pathway (Miller et al., 1987); therefore, processes much slower than diffusion up to the channel mouth limit the rate of Ba^{2+} binding. Figure 4 displays raw records of single channels in the presence of 4 m*m* external Ba^{2+}, under control conditions and with 1 *m* sucrose present. The records qualitatively show that high external sucrose concentration has no effect on the Ba^{2+} block kinetics. In a series of experiments under similar conditions, this conclusion was confirmed quantitatively; the ratios of block times and unblock times in the presence of 1

Table II: Effect of Ionic Strength on CTX Kinetics[a]

[KCl] (M)	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	relative sucrose effect
20	2×10^8	0.061	ND[b]
150	5×10^6	0.077	2.6 ± 0.1
800	2×10^5	0.14	2.5 ± 0.1

[a] CTX block kinetics were measured on single channels at different ionic strengths. All experimentss used 150 mM KCl in the isnternal medium, while KCl was included in the external solution at the indicated concentration. For 150 and 800 mM KCl, the relative effect of 1 *m* sucrose on the mean block time, as in Figure 3A, is also reported. Data represent averages of three to eight separate measurements. [b] Not determined.

m sucrose to those in its absence were 0.98 ± 0.03 and 1.2 ± 0.1, respectively (mean ± SEM of three experiments). This control experiment further supports the idea that the slowing of CTX kinetics in solutions containing sugars is due to increased viscosity, and not to nonspecific damage of the channel protein.

The binding of CTX to the Ca^{2+}-activated K^+ channel is sensitive to ionic strength. As shown in Table II, the second-order association rate constant is high at low ionic strength and falls about 1000-fold as ionic strength approaches 1 M. Over this same range, the dissociation rate constant increases only about 2-fold. The profound lowering of the on-rate by increasing ionic strength is fully understandable as an effect of local electrostatic fields near the channel's external mouth (MacKinnon et al., 1989); the local concentration of the positively charged CTX near the channel's mouth is enhanced by negative charge on the channel protein, and this electrostatic effect is screened at high ionic strength. It is worth mentioning that the concept of "local concentration" is valid under the conditions here, since all rate measurements were made by a method based on spontaneous fluctuations at thermodynamic equilibrium. Since the on-rate constant at 800 mM salt, $\sim 10^5$ M^{-1} s^{-1}, is 3–4 orders of magnitude below a typical value for a diffusion-controlled rate, we might expect that viscosity dependence should be lost at high salt. However, this is not the case (Table II). Sucrose is just as effective in slowing the CTX kinetics at high salt as it is under standard conditions. Therefore, diffusion remains the rate-limiting step in toxin binding even at high salt concentrations when the absolute on-rate is low.

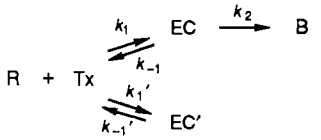
Discussion

These results provide strong evidence that aqueous diffusion provides the rate-controlling step for CTX binding to its receptor in the mouth of the Ca^{2+}-activated K^+ channel. The precisely inverse relation between solution viscosity, as manipulated by three different solutes, and CTX kinetics is a required prediction of a fully diffusion-controlled process; for such a result to arise for other reasons, such as effects of solvent composition on the channel structure, a most unlikely set of circumstances would be required, especially since the solvent conditions employed do not affect channel gating kinetics, a notoriously finicky process. It is important to realize that in any diffusion-controlled process, solution viscosity must alter off-rate as well as on-rate (Schurr, 1970a); solution viscosity will affect the ligand's likelihood of diffusing far enough away from the receptor to avoid being recaptured, just as it influences diffusion of a ligand from bulk solution up to the receptor. Since these strong predictions are confirmed here, we may accept the idea that CTX binding is diffusion-controlled.

At physiological ionic strength, the CTX on-rate is respectably high, as might be expected for a diffusion-controlled protein–protein interaction, on the order of 10^7 M^{-1} s^{-1}. However, this value can be misleading, since the association rate of CTX is very strongly ionic strength dependent (Anderson et al., 1988). The value of the on-rate at high salt concentrations, where local surface potentials are screened, is in fact much lower, on the order of 10^5 M^{-1} s^{-1} (Table II). This rate constant is several orders of magnitude lower than typical values for diffusion-controlled rates, and is well below the value that has been claimed to be an absolute lower limit for diffusion-limited reactions (Schurr, 1970b). It is therefore worthwhile to address the following question: given that CTX binding is diffusion-controlled, how can the absolute on-rate be so low?

One way to account for this would be to propose that nearly all the time the channel is in a CTX-insensitive state and that only a very rare conformation of the K^+ channel is competent to bind CTX. This idea cannot be ruled out, but it is unattractive because there is no independent evidence to suggest the existence of such a CTX-insensitive conformation. Indeed, it is known that CTX can bind to at least two distinguishable conformations of the channel, both the open and closed states, and that dissociation rates from these two states are equal (Anderson et al., 1988).

If we assume the K^+ channel to be always available for CTX block, we can attack the above question in a more interesting way. First, we must realize that a diffusion-controlled on-rate does *not* indicate that every collision of the toxin with the channel must lead to productive binding, as is sometimes asserted (Olea & Thomas, 1988). Instead, if only a small fraction of toxin collisions lead to the bound state, then the process can still be viscosity-dependent, but will display a low association rate. To see this, consider the kinetic model below, in which we assume that rates of toxin binding are diffusion-controlled and that, therefore, $k_2 \gg k_{-1}$ (Schurr, 1970a):



Here, we assume that in order for the toxin (Tx) and receptor (R) to attain a productively bound state, B, an encounter complex, EC, of just the right molecular configuration must first form; all other toxin–receptor encounters, labeled EC′ here, cannot proceed to the bound state. If EC represents the rare encounter complex formed by a correctly oriented toxin molecule, for example, then EC′ would represent the large set of complexes in which the toxin is incorrectly oriented. Let k_{dif} represent the "Smoluchowski diffusion limit", i.e., the second-order rate constant (in units of liters per mole per second) for all collisions of the toxin and receptor (Schurr, 1970a; Rice, 1985); then

$$k_1 + k_1' = k_{dif} = 4\pi r D N_0 / 1000 \quad (4)$$

where D is the bulk diffusion coefficient of the toxin, r is the "capture radius" of the toxin–receptor complex, and N_0 is Avogadro's number. If p is the probability of the toxin diffusing up to the channel in just the correct orientation to form a productive encounter, we have

$$k_{on} = k_1 = p k_{dif} \quad (5)$$

Since by assumption $k_2 \gg k_{-1}$, k_{on} will still be viscosity dependent, but now its value can be much lower than the Smoluchowski limit. For example, suppose that the externally facing mouth of the channel is 5 Å wider than the girth of the toxin (i.e., capture radius of 2.5 Å). Then, given a toxin diffusion coefficient of 10^{-6} cm^2/s, the expected Smoluchowski

diffusion-controlled on-rate would be on the order of 10^8 M^{-1} s^{-1}. However, if the toxin, upon entering the mouth, were "correctly" oriented only 0.1% of the time, then the measured on-rate constant would be only 10^5 M^{-1} s^{-1}. According to this picture, the toxin attains its bound state in a conventional and unsophisticated way, by a process of blind trial and error. Indeed, in the approach of the toxin up to the channel receptor, there is no "molecular recognition" occurring at all; the toxin tries and fails to bind many times for every correct encounter.

An essential, and quite unusual, feature of this view is that once in an "incorrect" encounter complex, the toxin cannot attain the correct encounter complex without first diffusing away from the channel into bulk solution. In other words, the toxin, once in intimate but nonproductive contact with the receptor, cannot seek out a correct orientation while remaining in close contact; it cannot "spin around" within an encounter complex. If such hunting through configuration-space could occur, then incorrect encounters would be rapidly converted to productive encounters, and the on-rate would approach the Smoluchowski limit.

As mentioned above, the interaction of CTX with the Ca^{2+}-activated K^+ channel is strongly electrostatic. The entryway of the channel carries carboxylate residues which set up a negative electrostatic potential locally near the CTX binding site, and thereby promote entry of the positively charged toxin (MacKinnon & Miller, 1989; MacKinnon et al., 1989). This effect can be formally considered either as an increase in the local concentration of the toxin or as an increase in capture radius of the channel mouth (eq 4). Within the framework of either formal viewpoint, the influence of local electrostatics should decrease with the ionic strength of the medium. For this channel, local electrostatic effects are almost fully suppressed above 500 mM external ionic strength (Anderson et al., 1988; MacKinnon & Miller, 1988). We see here (Table II) that the toxin binding rate is about 20-fold faster at physiological ionic strength than the rate at 800 mM KCl, which is a good approximation to the inherent rate in the absence of local electrostatic fields. The binding reaction is equally viscosity-sensitive across the entire ionic strength range, as is demanded by the present model.

These results provide an example of "stupid" molecular recognition between a peptide neurotoxin with its ion channel receptor. According to this view, the peptide blocker must approach its receptor in precisely the correct orientation for productive binding to occur. Local electrostatic forces lure toxin molecules toward the receptor, but do not explicitly orient them, as has been proposed in other molecular recognition reactions (Getzoff et al., 1983; Sharp et al., 1987; Northrup et al., 1988). This model requires that a CTX molecule, near the binding site but in an incorrect orientation, is so constrained in its movements that it cannot reorient without first dissociating into solution. This picture is fully consistent with the proposal (MacKinnon & Miller, 1988, 1989) that the toxin receptor is located in a "mouth" or "vestibule" of the channel, where multiple interactions between protein residues are made over an extended area; it is further in harmony with the recent 2-D NMR structure determination of CTX (Massefski et al., unpublished results), which shows groups known to affect binding to be separated by distances as large as 2.5 nm. Using a synthetic gene for CTX expressed at high levels in *Escherichia coli*, we are presently working to identify groups on the peptide which are directly involved in recognition, however stupid, by the K^+ channel receptor.

ACKNOWLEDGMENTS

I am grateful for the excellent experimental facilities and scientific services at the Marine Biological Laboratories, Woods Hole, MA, where the majority of this work was performed. I am particularly indebted to Drs. W. P. Jencks and R. MacKinnon for trenchant suggestions throughout the course of this work, and to Dr. C. Smith for criticism of the manuscript.

REFERENCES

Anderson, C., MacKinnon, R., Smith, C., & Miller, C. (1988) *J. Gen. Physiol. 91*, 317–333.

Barrett, J. N., Magleby, K. L., & Pallotta, B. S. (1982) *J. Physiol. 331*, 211–230.

Benham, C. D., Bolton, T. B., Lang, R. J., & Takewaki, T. (1985) *Pflugers Arch. 403*, 120–127.

Brouwer, A. C., & Kirsch, J. F. (1982) *Biochemistry 21*, 1302–1307.

Gates, C. A., & Northrop, D. B. (1988) *Biochemistry 27*, 3834–3842.

Getzoff, E. D., Tainer, J. A., Weiner, P. K., Kollman, P. A., Richardson, J. A., & Richardson, D. C. (1983) *Nature (London) 306*, 287–290.

Golowasch, J., Kirkwood, A., & Miller, C. (1986) *J. Exp. Biol. 124*, 5–13.

Grinstein, S., & Smith, J. D. (1989) *Am. J. Physiol. 257*, C197–C206.

Guha, M. K., VanderJagt, D. L., & Creighton, D. J. (1988) *Biochemistry 27*, 8818–8822.

Hermann, A., & Erxleben, C. (1987) *J. Gen. Physiol. 90*, 27–47.

Lee, J. C., & Timasheff, S. N. (1981) *J. Biol. Chem. 256*, 7193–7201.

Lim, W. A., Raines, R. T., & Knowles, J. R. (1988) *Biochemistry 27*, 1158–1167.

Lucchesi, K., Ravindran, A., Young, H., & Moczydlowski, E. (1989) *J. Membr. Biol. 109*, 269–281.

MacKinnon, R., & Miller, C. (1988) *J. Gen. Physiol. 91*, 335–349.

MacKinnon, R., & Miller, C. (1989) *Biochemistry 28*, 8087–8092.

MacKinnon, R., Reinhart, P. H., & White, M. M. (1988) *Neuron 1*, 997–1001.

MacKinnon, R., Latorre, R., & Miller, C. (1989) *Biochemistry 28*, 8092–8099.

Miller, C. (1988) *Neuron 1*, 1003–1006.

Miller, C., & White, M. M. (1980) *Ann. N.Y. Acad. Sci. 341*, 534–551.

Miller, C., Moczydlowski, E., Latorre, R., & Phillips, M. (1985) *Nature (London) 313*, 316–318.

Miller, C., Latorre, R., & Reisin, I. (1987) *J. Gen. Physiol. 90*, 427–449.

Moczydlowski, E., & Latorre, R. (1983) *J. Gen. Physiol. 82*, 511–542.

Moczydlowski, E., & Latorre, R. (1983) *Biochim. Biophys. Acta 732*, 412–420.

Moczydlowski, E., Alvarez, O., Vergara, C., & Latorre, R. (1985) *J. Membr. Biol. 83*, 273–282.

Northrup, S. H., Boles, J. O., & Reynolds, J. C. L. (1988) *Science 241*, 67–70.

Olea, A. F., & Thomas, J. K. (1988) *J. Am. Chem. Soc. 110*, 4494–4502.

Pittz, E. P., & Timasheff, S. N. (1978) *Biochemistry 17*, 615–623.
Ravindran, A., & Moczydlowski, E. (1989) *Biophys. J. 55*, 359–365.
Rice, S. A. (1985) *Comprehensive Chemical Kinetics*, Vol. 25, Elsevier Publishing Co., Amsterdam.
Sands, S. B., Lewis, R. S., & Cahalan, M. D. (1989) *J. Gen. Physiol. 93*, 1061–1074.
Schurr, J. M. (1970a) *Biophys. J. 10*, 700–716.
Schurr, J. M. (1970b) *Biophys. J. 10*, 717–727.
Schweitz, H., Bidard, J.-N., Maes, P., & Lazdunski, M. (1989) *Biochemistry 28*, 9708–9714.
Sharp, K., Fine, R., & Honig, B. (1987) *Science 236*, 1460–1463.
Smith, C. D. (1988) Ph.D. Thesis, Brandeis University.
Smith, C. D., Phillips, M., & Miller, C. (1986) *J. Biol. Chem. 261*, 14607–14613.
Valdivia, H. H., Smith, J. S., Martin, B. M., Coronado, R., & Possani, L. D. (1988) *FEBS Lett. 226*, 280–284.
Vergara, C., & Latorre, R. (1983) *J. Gen. Physiol. 82*, 543–568.
Weast, R. C. (1983) *CRC Handbook of Chemistry and Physics*, 64th ed., CRC Press, Boca Raton, FL.

Glu-69 of the D2 Protein in Photosystem II Is a Potential Ligand to Mn Involved in Photosynthetic Oxygen Evolution†

Wim Vermaas,* Jeroen Charité,‡ and Gaozhong Shen
Department of Botany and the Center for the Study of Early Events in Photosynthesis, Arizona State University, Tempe, Arizona 85287-1601

Received November 28, 1989; Revised Manuscript Received January 31, 1990

ABSTRACT: To probe the involvement of amino acid residues of the D2 protein in the water-splitting process in photosystem II, site-directed mutagenesis was applied to identify D2 residues that might contribute to binding the Mn cluster involved in oxygen evolution. Mutation of Glu-69 to Gln or Val in D2 of the cyanobacterium *Synechocystis* sp. PCC 6803 was found to lead to a loss of photoautotrophic growth. However, in cells of the Gln mutant (E69Q) a significant Hill reaction rate could be observed upon the start of illumination, but the oxygen evolution rate declined with a half-time of approximately 1 min. Addition of 1 mM Mn^{2+} stabilized oxygen evolution in E69Q thylakoids. Other divalent cations were ineffective in specific stabilization. When the water-splitting system was bypassed, the rate of electron transport remained stable during illumination, indicating that the inactivation of oxygen evolution is localized in the water-splitting complex. We interpret these observations to indicate that Glu-69 is a Mn ligand and that the loss of oxygen evolution in the E69Q mutant upon turnover of PS II is initiated by changes in the Mn cluster, possibly leading to Mn release from the water-splitting complex. The addition of exogenous Mn to E69Q thylakoids may help to keep the Mn cluster active for a longer time, perhaps by providing Mn to rebind in the cluster after release of one Mn and before the Mn cluster had disintegrated. The Glu-69 residue is conserved in D2 of higher plants, and Glu and Asp residues are present in D1 at locations potentially homologous to that in D2; such D1 residues may also be involved in providing a proper binding environment to the Mn cluster.

Oxygen evolution is one of the most intriguing "black boxes" in the process of photosynthesis. One molecule of oxygen is evolved upon every four single-electron turnovers of the photosystem II (PS II) complex, with an oxidizing equivalent accumulating in the oxygen-evolving system with each turnover of PS II, until sufficient equivalents have accumulated to oxidize 2 molecules of water [reviewed by Babcock (1987) and Rutherford (1989)].

The oxidizing equivalents are stored in the oxygen-evolving complex, and a (presumably tetranuclear) Mn cluster plays a major role in accumulating these equivalents. It is unknown with which proteins of the PS II complex the Mn cluster primarily interacts, but there are a number of observations suggesting that the reaction center protein D1 and perhaps also D2 are involved in Mn binding. It has been shown that at least part of Mn binding is impaired in a mutant that does not process the C-terminal end of D1 (Metz et al., 1986; Diner et al., 1988a; Seibert et al., 1988), thus suggesting that the C-terminal end of D1 interacts, directly or indirectly, with the Mn cluster. Also, the realization that the donors D and Z (which interact with the Mn cluster) are Tyr residues in D2 and D1, respectively (Debus et al., 1988a,b; Vermaas et al., 1988a; Metz et al., 1989), has enhanced the notion that the Mn cluster may be closely associated with D1 and D2. On the basis of the sensitivity of the magnetic relaxation kinetics of EPR signals associated with D and Z to the presence of Mn it has been estimated that the distance between Z and the Mn cluster is considerably shorter than that between D and Mn (Babcock, 1987), possibly implying that the interaction of the Mn cluster is stronger with D1 than with D2. However, it should be kept in mind that the calculations on which these estimates are based are dependent on the (unknown) magnetic properties of the medium between the various paramagnetic species and that the actual distances could be significantly different from these estimates. Also, as was discussed by Rutherford (1989), the average redox state of the Mn cluster was not identical for the measurements of the D^+/Mn vs

† This research was supported by NSF (DCB87-16055).
‡ Present address: Hubrecht Laboratory, Netherlands Institute for Developmental Biology, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands.

0006-2960/90/0429-5325$02.50/0