Articles

Mapping Function to Structure in a Channel-Blocking Peptide: Electrostatic Mutants of Charybdotoxin†

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ABSTRACT: Electrostatic interactions between charybdotoxin (CTX), a specific peptide pore blocker of K^+ channels, and a Ca^{2+}-activated K^+ channel were investigated with a genetically manipulable recombinant CTX. Point mutations at certain charged residues showed only small effects on the binding affinity of the toxin molecule: Lys11, Glu12, Arg19, His21, Lys31, and Lys32. Replacement by Gln at Arg25, Lys27, or Lys34 strongly decreased the affinity of the toxin. These affinity changes were mainly due to large increases of toxin dissociation rates without much effect on association rates, as if close-range interactions between the toxin and its receptor site of the channel were disrupted. We also found that the neutralization of Lys27 to Gln removed the toxin's characteristic voltage dependence in dissociation rate. Mutation and functional mapping of charged residues revealed a molecular surface of CTX which makes direct contact with the extracellular mouth of the K^+ channel.

Many K^+-selective ion channels are specifically inhibited by a family of neurotoxic peptides found in scorpion venoms (Moczydlowski et al., 1988). These peptide toxins, which are 36–38 residues long and share strong sequence similarity among many isoforms, block with nanomolar affinity both voltage-gated and Ca^{2+}-activated K^+ channels (Carbone et al., 1982; Moczydlowski et al., 1988; Possani et al., 1982; Gimenez-Gallego et al., 1988; Smith et al., 1986; Galvez et al., 1990; Wolff et al., 1988; Schweitz et al., 1989; Price et al., 1989; Vazquez et al., 1990). The mechanism by which one of these, charybdotoxin (CTX),[1] blocks Ca^{2+}-activated K^+ channels has been studied in depth. It is known (MacKinnon & Miller, 1988) that a single CTX molecule physically occludes the K^+ conduction pathway by binding to a receptor located in the externally facing "mouth" of the channel protein. Because the peptide's inhibitory mechanism is simple and its receptor site is located in a mechanistically important region of the ion channel, CTX is a potentially useful structural probe of K^+ channels. The solution structure of CTX has recently been determined (Bontems et al., 1991, 1992), and it will now be especially informative to identify the residues involved in the toxin's channel blocking function.

Charybdotoxin is a highly basic protein, with eight positively charged residues (four lysines, three arginines, and 1 histidine), two negatively charged groups (a single glutamate and the C-terminus), and a net charge of +5 to +6 at neutral pH (Gimenez-Gallego et al., 1988). Electrostatic forces are known to favor CTX binding to the negatively charged mouths of K^+ channels (Anderson et al., 1988; MacKinnon & Miller, 1989; MacKinnon et al., 1989). This report investigates these charged residues to assess which are directly involved in intimate toxin channel contact and which are inessential for forming a tight complex. Our approach is a conventional one: to neutralize each of these groups and to examine the resulting changes in the toxin's association–dissociation kinetics with single K^+ channels. We use site-specific mutations of a gene coding for a fusion protein which can be proteolytically cleaved to release functionally active CTX (Park et al., 1991).

We find that these electrostatic mutations lower the toxin's association rate by only small factors and that alterations at three positions, Arg25, Lys27, and Arg34, give rise to large changes in dissociation rate. These three residues are located closely on one side of the CTX molecule. On the opposite side are located five other charged residues, Lys11, Gly12, Arg19, Lys31, and Lys32, which may be neutralized with only small alterations of the toxin's off-rate. We conclude that positively charged groups on CTX promote toxin channel interaction in two ways: by weak, through-space electrostatic influences and by direct and intimate contact with the channel on one side of the toxin molecule.

MATERIALS AND METHODS

Materials. Restriction enzymes and DNA ligase were purchased from New England Biolabs (Beverly, MA), Taq DNA polymerase and the DNA amplification kit were from Perkin-Elmer Cetus (Norwalk, CT), and bovine blood restriction protease factor X_a was from Boehringer Mannheim Biochemicals (Indianapolis, IN). Phospholipids were purchased from Avanti Polar Lipids (Birmingham, AL).

Site-Directed Mutagenesis of Recombinant CTX Using the Polymerase Chain Reaction. Point mutagenesis of the CTX coding sequence residing in pCSP105 (Park et al., 1991) was achieved by mismatch amplification using two sequential polymerase chain reactions (PCRs) (Kammann et al., 1985). The entire CTX gene and flanking sequences were amplified using mutagenic primers containing the desired sequences for mutation and extending about 15 bases on each side of the mismatch region. Flanking primers (20-mers) recognized

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[1] Abbreviations: CTX, charybdotoxin; PCR, polymerase chain reaction; DEAE, diethylaminoethyl; FPLC, fast-performance liquid chromatography; HPLC, high-performance liquid chromatography; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenebis-(oxyethylenenitrilo)]tetraacetic acid; TEA, tetraethylammonium.

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sequences outside the *Sal*I and *Hin*dIII restriction sites of pCSP105. Amplification was carried out in a DNA thermal cycler for 30 cycles of 1.5-min denaturation at 94 °C, 1-min annealing at 40 °C, and 1.5-min polymerization at 68 °C. The reaction mixture for the first PCR contained 50 μL of 10 mM Tris-HCl, pH 8.3/50 mM KCl/1.5 mM $MgCl_2$/300 μM dNTP/0.01% gelatin/2 units of Taq polymerase/40 pg of plasmid DNA/100 pM each of primer. The conditions for the second PCR were identical, except that 5 μL of the first PCR product and a 100 pM quantity of the second flanking primer were added.

The amplified DNA was cleaned by phenol extraction and ethanol precipitation and then digested with *Sal*I and *Hin*dIII. The 145-residue fragment was gel purified and inserted into the pCSP105 fusion vector to replace the wild-type recombinant CTX gene for later transformation. Candidate plasmids of mutant CTX were propagated in *Escherichia coli* DH1, and the entire DNA sequence of each mutant CTX gene was confirmed before use.

Expression, Purification, and Modification of Mutant CTX. Each mutant CTX was expressed as a cleavable fusion protein in *E. coli* strain BL21(DE3) and partially purified by ammonium sulfate precipitation and DEAE-cellulose chromatography as described previously (Park et al., 1991). The column eluate was dialyzed against 2 L of 50 mM Tris-HCl, pH 8.0/150 mM NaCl/0.5 mM 2-mercaptoethanol. After dialysis, 3 mM $CaCl_2$ and factor X_a (10 μg/mg of fusion protein) were added, and the digestion was allowed to proceed at room temperature for 30 h. The CTX peptide was purified by FPLC on a Mono-S column as described (Park et al., 1991). After cyclization of N-terminal glutamine, the peptide was rechromatographed on Mono-S and purified to homogeneity by reversed-phase HPLC (C_{18}, Vydac, 4.6 × 250 nm, 5 μm). The concentration of mutant CTX in the final preparation was determined by absorbance at 280 nm, using the extinction coefficient of wild-type recombinant CTX, ϵ = 10 000 M^{-1} cm^{-1} (Park et al., 1991); amino acid analysis of three of the mutant toxins confirmed this extinction coefficient for mutants not involving aromatic residues.

Functional Assay of Mutant CTX in Planar Bilayers. The channel blocking activity of each mutant toxin was quantified on single high-conductance Ca^{2+}-activated K^+ channels from rat skeletal muscle inserted into planar lipid bilayers, as described previously (Anderson et al., 1988). Single channels were incorporated into planar bilayers formed from a decane solution of 85% 1-palmitoyl-2-oleylphosphatidylethanolamine/15% 1-palmitoyl-2-oleoylphosphatidylcholine by addition of plasma membrane vesicles under osmotic gradient conditions. After a single channel was inserted, further channel insertion was suppressed by removing the salt gradient, such that 150 mM KCl was present on both sides of the membrane.

Blocking and dissociation kinetics of each CTX variant were measured from at least 100 discrete blocking events caused by the addition of peptide to the external side of the bilayer. The final solutions on the two sides of the membrane were as follows, unless otherwise specified: internal solution, 10 mM HEPES, pH 7.4/150 mM KCl/30 μM $CaCl_2$; external solution, 10 mM HEPES, pH 7.4/150 mM KCl/0.1 mM EGTA/30 μg/mL bovine serum albumin/5–1000 nM toxin. To obtain the dissociation rate at low internal K^+ concentration, the internal chamber was thoroughly perfused with 10 mM HEPES, pH 7.4/3 mM KCl/147 mM NaCl/30 μM $CaCl_2$; at low K^+, the dissociation rate was voltage independent and was typically checked at two to three voltages between 20 and 50 mV. For experiments involving variation of ionic strength, both sides of the bilayer were brought to 50 mM KCl after channel incorporation, and the external ionic strength was increased to the desired value by addition of NaCl. In the tetraethylammonium (TEA) competition experiments, control records without TEA were collected in the presence of known concentrations of toxin to determine the mean burst time, mean block time, and the single-channel current. Then, TEA was added to the external side of the bilayer up to 1.2 mM, and the measurements were repeated, as previously described (Miller, 1988).

Data Collection and Analysis. The voltage clamp circuit for measuring single-channel currents and the data acquisition system were as described previously (Anderson et al., 1988). The association and dissociation of CTX variants were determined from the statistical distributions of discrete blocked and unblocked dwell times measured directly from the single channel records. Blocking events were recognized unambiguously as nonconducting intervals longer than 300 ms for all mutant toxins except K27Q, since channel closing events were short-lived with an average dwell time in the range of 1–10 ms. For K27Q, a 30-ms interval was used as the cutoff limit, and the channel open probability was always adjusted with Ca^{2+} to be >0.8, to ensure that spontaneous channel closings would be much shorter-lived than toxin-induced blocking events. Apparent rate constants of association and dissociation were measured from the mean blocked and unblocked times, τ_b and τ_u, respectively:

$$k_{on} = \tau_u^{-1}[CTX]^{-1} \quad (1)$$

$$k_{off} = \tau_b^{-1} \quad (2)$$

The apparent dissociation constant for each derivative was calculated from the measured probability of channel block, p_b, at a given toxin concentration:

$$K_d = [CTX](1 - p_b)/p_b \quad (3)$$

RESULTS

To assess the functional consequences of point mutations of CTX, we used a high-resolution assay system: the blocking of single Ca^{2+}-activated K^+ channels by individual CTX molecules (Anderson et al., 1988). Single high-conductance Ca^{2+}-activated K^+ channels from rat skeletal muscle plasma membranes were reconstituted into planar lipid bilayers; addition of CTX to the external solution induces long-lived "blocked" intervals (Figure 1A) that represent the binding of individual toxin molecules to the single channel (Miller et al., 1985). The association and dissociation rate constants, k_{on} and k_{off}, may be directly determined from records such as these, since it is well-established that toxin channel interaction is strictly bimolecular (Smith et al., 1986; Anderson et al., 1988):

$$\text{CTX} + \text{channel} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \text{CTX–channel}$$

The statistical distributions of blocked and unblocked times yield both association and dissociation rates of the toxin at thermodynamic equilibrium (Moczydlowski et al., 1984; Miller et al., 1985). This method allows unequivocal dissection of toxin binding into association and dissociation rates and thus reveals those mutations which affect the stability of toxin already on the receptor site.

To produce charge-altered CTX mutants, we used the cleavable fusion protein strategy described recently for

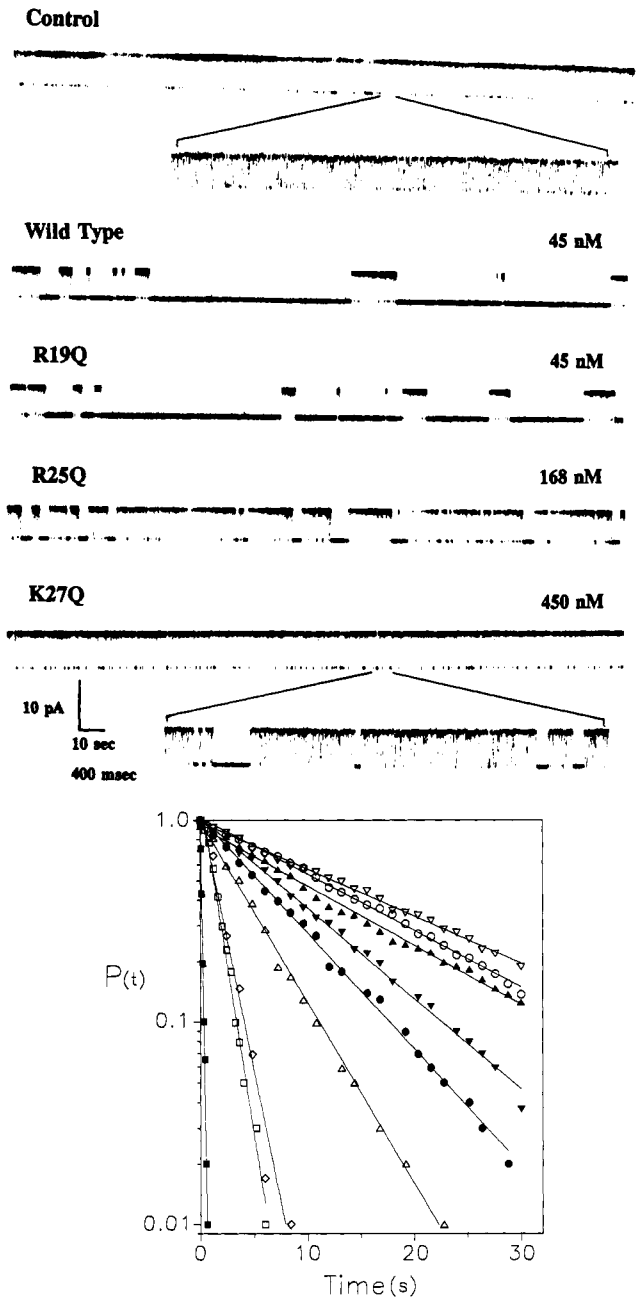

FIGURE 1: K^+ channel block by CTX mutants. (A, top) Discrete K^+ channel blocking events by CTX variants. Single Ca^{2+}-activated K^+ channels were inserted into planar lipid bilayers, and discrete blocking events were induced by the addition of the indicated toxin to the external side of the bilayer. Channel openings in this and all other data traces are upward. (B, bottom) Cumulative probability distribution of CTX mutant block times. The probability, $P(t)$, that dwell time in the blocked state is longer than time t was calculated from at least 100 blocking events for K27Q (■), R25Q (□), R34Q (◇), K31Q (△), K11Q (●), H21Q (▼), K32Q (▲), R19Q (▽), and wild-type (○) CTX.

expressing fully active CTX in *E. coli* (Park et al., 1991). All altered toxins were active as K^+ channel blockers, gave final yields similar to that of wild-type CTX (0.5–1.0 mg/L of bacterial culture), and displayed similar chromatographic profiles during purification (Table I).

Mutational Mapping of Positively Charged Residues. The strong ionic strength dependence of the CTX block of Ca^{2+}-activated K^+ channels shows that electrostatic forces contribute to the binding of this very basic peptide (Anderson et al., 1988; MacKinnon et al., 1989; Miller 1990). But it is a priori unlikely that all of the charged groups on the toxin contribute equally to the toxin binding. We therefore changed each charged residue individually to a neutral polar group, glutamine, and observed the effect of this alteration on CTX blocking characteristics. Raw single-channel traces of several of these CTX derivatives are shown in Figure 1A. All induce clear blocked intervals in the single-channel records; in all cases, the distributions of blocked-state dwell times are single exponential (Figure 1B), as demanded for a single molecular species of blocker binding to the channel with bimolecular kinetics (Moczydlowski et al., 1986). Dwell times of the unblocked channel are also exponentially distributed (data not shown).

The single-channel records of Figure 1 illustrate that charge alterations produce very different effects at different positions. Table I summarizes the blocking characteristics of all mutants studied, which vary in binding affinity over a 500-fold range. All charge-neutralizing mutations lower the association rate constant by small factors (1.1–5-fold), as expected if a through-space electrostatic effect operates in concentrating the blocker in the vicinity of the channel mouth (Bell & Miller, 1984; MacKinnon et al., 1989). Three residues, Glu12, Arg19, and Lys32, may be neutralized without significant effect on toxin binding kinetics. Charge neutralizations at Lys11, His21, and Lys31 result in moderate (3–5-fold) weakening in affinity. Dramatic decreases in affinity, mainly due to large increases of off-rate, were seen at the three residues, Arg25 (41-fold), Lys27 (500-fold), and Arg34 (22-fold). The average lifetime of the weakest of these, K27Q, on its binding site was about 100 ms, as compared to 16 s for wild-type CTX. Neutralization of Lys27 with Asn also destabilizes toxin binding, but less drastically (6-fold increase in off-rate) than with Gln.

Effect of Toxin Charge on Association Rate. The association of CTX with the channel is strongly dependent on ionic strength (Anderson et al., 1988; MacKinnon et al., 1989), as expected for the interaction of a highly basic toxin with a negatively charged receptor site. The weakening of CTX binding with ionic strength is manifested almost wholly in the association rate, which falls 1000-fold over a 30-fold range of ionic strength, while the off-rate increases only 2-fold. The capability of specific mutagenesis of the toxin allows a closer look at the contribution of electrostatic forces to CTX binding. We studied a CTX variant with two fewer positive charges than native toxin by constructing the double mutant R19Q/K32Q.

We tested a simple expectation of surface electrostatics: that the on-rate of a mutant with reduced net charge (+3.5 vs +5.5 for native CTX) should be lower than that of CTX and should also be less sensitive to ionic strength (MacKinnon et al., 1989). We have seen that the on-rates of all reduced-charge mutants are slightly lowered under normal assay conditions (150 mM salt). Figure 2 presents the ionic strength variation in blocking kinetics of the double mutant R19Q/K32Q. As expected for this cationic toxin interacting with the anionic channel, the on-rate decreases with ionic strength, while the off-rate increases only slightly (Figure 2, inset). However, the reduced-charge mutant displays a quantitative surprise: its on-rate shows about the same, not less, sensitivity to ionic strength as CTX. This behavior, though quantitatively subtle, is a violation of the expectations of simple electrostatic theory.

Voltage Dependence of Toxin Dissociation. In the presence of K^+ in the internal aqueous medium, the CTX dissociation rate is voltage dependent, with the off-rate increasing as voltage is made increasingly positive inside (Anderson et al., 1988). This voltage dependence arises from K^+ ions traversing the channel's conduction pore from inside and destabilizing CTX

Table I: Chromatographic and Kinetic Parameters of Mutant CTX[a]

	Mono-S FPLC elution concn (mM NaCl)	RP-HPLC elution time (min)	dissociation constant, K_d (nM)	association rate, k_{on} ($\times 10^{-6}$ M^{-1} s^{-1})	dissociation rate, k_{off} (s^{-1})
WT	335	27.8	15 ± 1	4.5 ± 0.2	0.067 ± 0.003
K11Q	295	27.2	56 ± 6	2.0 ± 0.1	0.111 ± 0.004
R19Q	305	28.5	13.8 ± 0.8	3.9 ± 0.3	0.056 ± 0.001
H21Q	330	26.3	71 ± 5	1.8 ± 0.3	0.13 ± 0.01
R25Q	250	27.9	610 ± 60	0.91 ± 0.09	0.71 ± 0.03
K27Q	230	27.5	7500 ± 700	1.21 ± 0.04	9.1 ± 0.5
K31Q	255	25.0	60 ± 5	3.2 ± 0.2	0.19 ± 0.01
K32Q	285	28.4	27 ± 4	2.7 ± 0.2	0.062 ± 0.002
K34Q	285	25.3	330 ± 10	1.4 ± 0.1	0.58 ± 0.01
K11N/E12Q	330	26.5	37 ± 3	3.1 ± 0.2	0.12 ± 0.01
K27N	235	27.3	79 ± 2	4.7 ± 0.4	0.37 ± 0.02
R19Q/K32Q	225	28.7	20 ± 3	3.6 ± 0.5	0.053 ± 0.005

[a] Each mutant toxin was purified on a Mono-S FPLC column equilibrated with 30 mM NaP_i with a linear gradient of 0–0.6 M NaCl and on a C_{18} reversed-phase HPLC column with a linear gradient of 0–30% acetonitrile over 30 min. Each rate constant represents the mean ± SE of independent determinations of at least three single channels, each in a separate bilayer held at +35 mV. Channel open probabilities were clamped between 0.5 and 0.7 with internal Ca^{2+}, except for K27Q, for which open probability was clamped to 0.8–0.9.

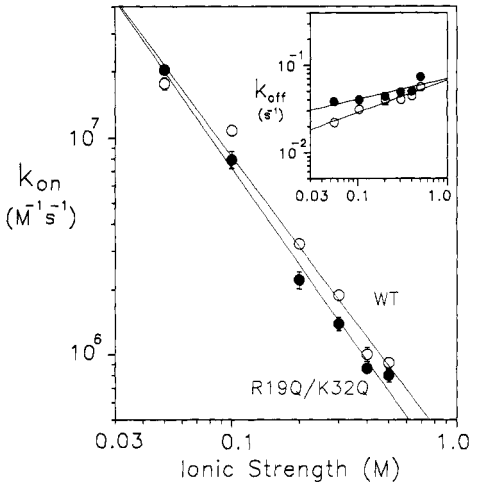

FIGURE 2: Influence of ionic strength on a reduced-charge mutant CTX. Association rate constants (k_{on}) and dissociation rates (k_{off}, inset) were measured as a function of external ionic strength for wild-type (○) and double mutant R19Q/K32Q (●) CTX, at symmetrical 50 mM KCl, with a holding voltage of 35 mV. Each point represents the mean ± SEM of four independent determinations.

on its site, possibly by electrostatic repulsion (MacKinnon & Miller, 1988). We therefore measured off-rates as a function of voltage to assess whether this indicator of peptide–K^+ ion interaction within the channel protein is preserved in the charge mutants. For example, the mean block time for R19Q lengthens about 4-fold if the membrane voltage is changed from +55 to +15 mV (Figure 3A). Figure 3B presents these dissociation rates, normalized to the "basal" dissociation rate measured with low K^+ present in the internal medium (MacKinnon & Miller, 1988). With internal K^+ present, all mutants except K27Q showed clear increases in off-rate with voltage, as with wild-type CTX, although some quantitative differences in voltage dependence were seen. For instance, R34Q showed weaker voltage dependence than wild-type peptide. In the absence of internal K^+, toxin dissociation rates were independent of voltage, as shown previously with native CTX (MacKinnon & Miller, 1988). These results imply strongly that these toxins all behave according to the same blocking mechanism as CTX and hence that they interact at the same place on the K^+ channel.

The mutations at Lys 27, however, require further study. The dissociation rate of K27Q, the fastest of all tested, is completely independent of applied voltage, as is that of the higher-affinity mutant K27N (Figure 3). This result might mean that K27Q is no longer binding at the same site as the other blockers. We therefore tested whether K27Q displays another characteristic known for "correct" CTX block (Miller, 1988): strict competition with externally added tetraethylammonium (TEA). A pore blocker with kinetics much faster than the time response of our recording system, TEA manifests channel block by reducing the observed single-channel current. If toxin and TEA cannot simultaneously occupy the channel receptor, then the single-channel current and the toxin on-rate must vary identically with TEA concentration (Miller, 1988):

$$i([TEA])/i(0) = k_{on}([TEA])/k_{on}(0) = 1/(1 + [TEA]/K_i)$$

where K_i is the inhibition constant for TEA. We measured the effects of external TEA on the association and dissociation rates of K27Q. As shown in Figure 4, the required relations hold for K27Q, as for native CTX. Therefore, TEA interferes with K27Q binding competitively, as expected if the mutant is acting at a site close to the normal CTX receptor.

DISCUSSION

Our intention here has been to develop a functional map of CTX to identify those residues which are important for high-affinity block of Ca^{2+}-activated K^+ channels. The charged residues of CTX provide an obvious first focus for structure–function studies, since the peptide is highly basic and since it is known that electrostatic forces promote binding to the negatively charged "mouth" of the K^+ channel (Anderson et al., 1988).

In surveying CTX variants with altered charge side chains, we have found that mutant toxins with disrupted binding block poorly mainly because of increased dissociation rates, not decreased association rates. This result makes sense. Since CTX binding is diffusion controlled (Miller, 1990), neither binding nor dissociation involves an energetically significant transition state to be negotiated by the toxin. Accordingly, we can validly conclude that if a point mutation gives a large affinity change manifested mainly in the off-rate, then the residue involved makes direct, intimate contact with the toxin-binding site.

Criteria for Correct Folding of Mutant Charybdotoxins. The production of fully active CTX by *E. coli* requires three in vitro posttranslational modifications: proteolytic release of the toxin peptide from the fusion protein, cyclization of the N-terminal glutamine to form pyroglutamate, and oxidation of the six cysteines to form the correct disulfide bonds. While these three steps are known to proceed faithfully for CTX itself (Park et al., 1991), there is no guarantee that every

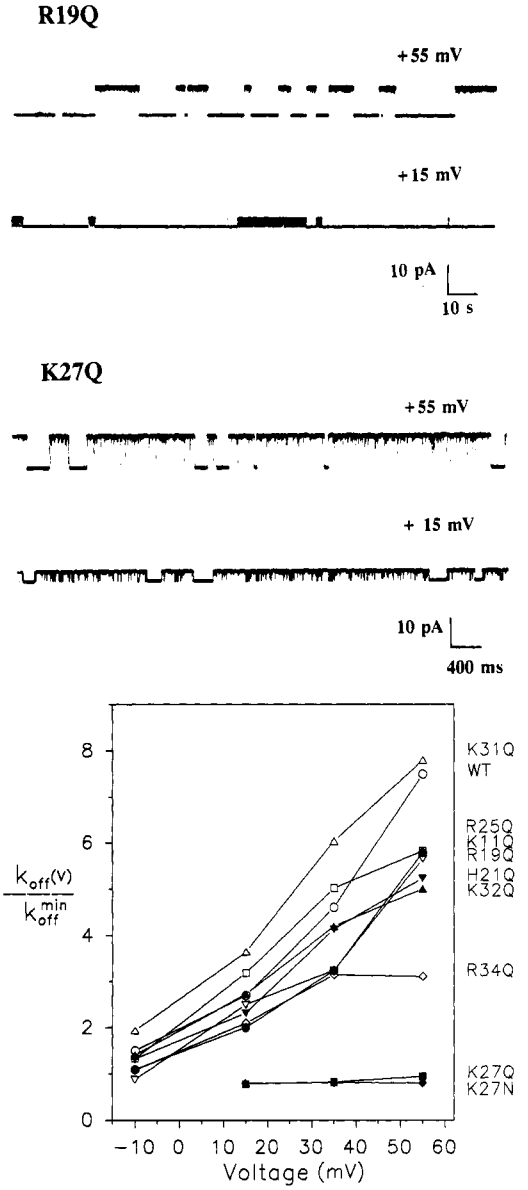

FIGURE 3: Voltage dependence of mutant CTX dissociation rate. (A, top) Block events by mutant toxins, R19Q and K27Q, recorded at two different membrane voltages, +55 and +15 mV. (Both inside and outside chambers contain 150 mM KCl.) (B, bottom) Dissociation rate (k_{off}) of each mutant toxin at different membrane voltages normalized to the voltage-independent basal dissociation rate measured with low K^+ (3 mM) in the internal solution, as described under Materials and Methods. Each point represents the mean value of three to six measurements from separate bilayers (SE, less than 10%).

variant will be so biochemically cooperative. We routinely monitored fusion protein cleavage and N-terminal cyclization, and we have not observed any differences in these processes between mutant and wild-type toxins. But we have no direct assay for correct disulfide bond formation. In interpreting the results here, we explicitly assume that folding and oxidation of mutant toxins proceed with full fidelity, as with wild-type CTX, i.e., that the concentration of peptide added in a given assay is identical to the concentration of functionally competent toxin. We consider this assumption justifiable on the basis of several lines of evidence.

First, the association rate constants of all mutant toxins are only slightly lower than that for native CTX. If only a small fraction of *E. coli*-expressed peptide were correctly folded, the apparent second-order rate constant for association would be correspondingly low. But this is not the case, as the low-

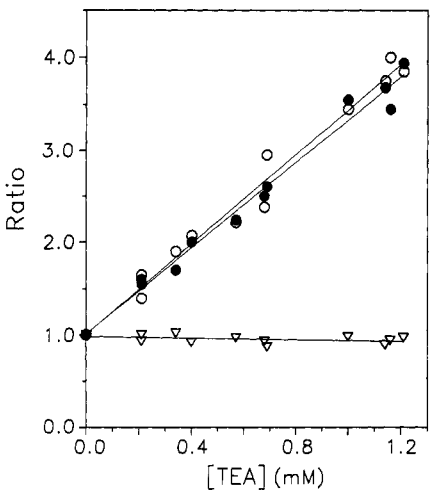

FIGURE 4: Effect of TEA on the channel block kinetics of the K27Q mutant. The single channel current, i, and the block kinetics of K27Q were measured before and after the addition of TEA. The ratios represent $\tau_u([TEA])/\tau_u(0)$ (●), $\tau_b([TEA])/\tau_b(0)$ (▽), and $i(0)/i([TEA])$ (○).

affinity mutants bind weakly not because of a low on-rate but rather because of high off-rates. Second, the yields and chromatographic behavior of CTX variants are similar to those for wild-type CTX. Though this is weak evidence, it is still worth mentioning because a mutant with a mixture of incorrectly folded forms would be expected to show complicated chromatographic profiles, especially in reversed-phase HPLC. Third, the block times of all toxins follow single-exponential distributions. This is an especially important fact, because it implies that, for each mutant, a single molecular species gives rise to the blocked state. Indeed, Ravindran and Moczydlowski (1989) showed that departure from a single-exponential block-time distribution is a particularly sensitive means of detecting impurities in a channel blocking preparation. Finally, we should mention that two-dimensional nuclear magnetic resonance (2-D NMR) spectra (not shown) of the lowest affinity mutant, K27Q, show clean backbone amide proton peaks at chemical shifts identical to those of native CTX (except, of course, Gln27); such a result would be extremely unlikely if our preparation contained a significant fraction of incorrectly disulfide-linked forms.

Functional Map of CTX. Figure 5 shows the van der Waals surface of CTX, as determined by 2-D NMR (Bontems et al., 1991, 1992), with all charged groups indicated. The molecule presents a lumpy, slightly oblate shape, with charged residues dispersed all over the molecular surface. The figure color codes the charged atoms according to the effect of charge neutralization by Gln substitution. Those residues showing large off-rate alteration are red (greater than 8-fold), while the "bland" residues (less than 3-fold increase in off-rate) are green. It is clear that the functionally important side chains, Arg25, Lys27, and Arg34, lie close together on the same side of the molecule, while the bland residues, Lys11, Glu12, Arg19, His21, and Lys32, occupy positions on the opposite side. This clear spatial segregation strongly suggests that the "base" of the CTX molecule makes direct contact with the channel mouth, while its "summit" does not. This proposal is strengthened by several known functionally disruptive chemical modifications of CTX. Iodination of Tyr36 (Lucchesi et al., 1989) and chymotrypsin cleavage at Phe2 (Smith et al., 1986) both speed the dissociation rate 20–50-fold, and both of these residues are located on the base of the molecule, close to Arg34. Site-directed mutants of nonpolar residues are currently being constructed to test this picture further.

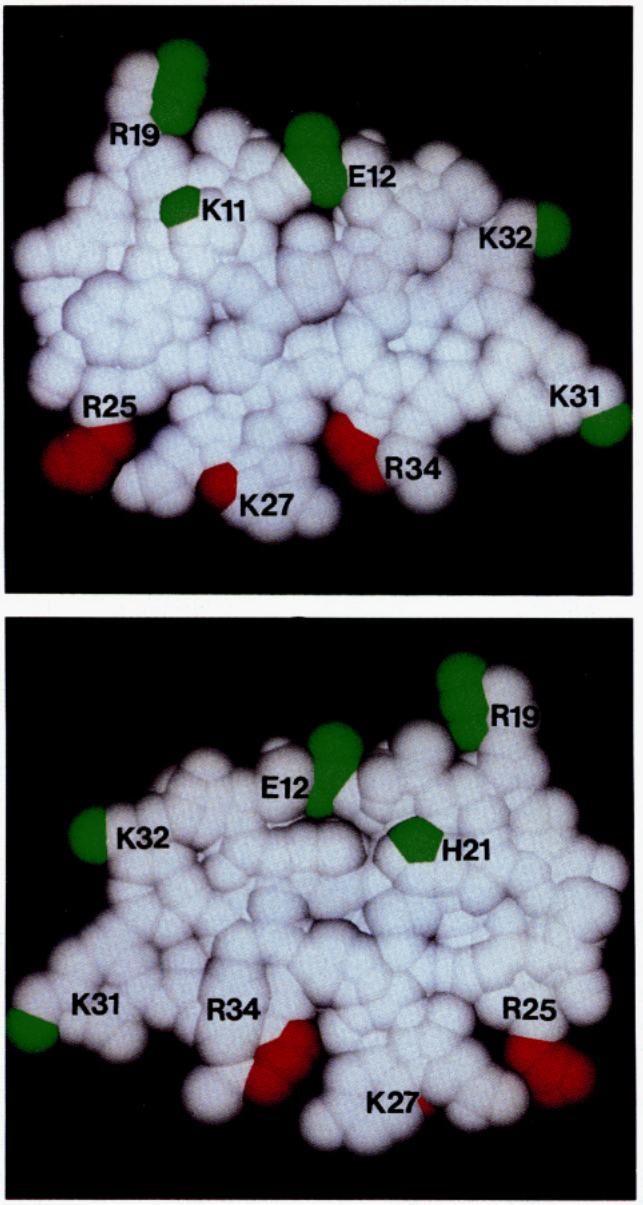

FIGURE 5: Functional map of the CTX molecule. The van der Waals surface for CTX, calculated from NMR-determined coordinates (Bontems et al., 1992), is shown with all charged atoms in Lys, Arg, His, and Glu residues colored according to the effects on the dissociation rate of replacement by Gln. Color code: red, greater than 8-fold increase in the off-rate; green, less than 3-fold increase. Upper and lower panels are rotated 180° about the *y*-axis.

Inadequacy of Simple Electrostatics. In interpreting our results, we are guided by the distinction between two types of forces that must be operating in the binding of a positively charged peptide to a negatively charged receptor. First, replacing a positively charged group with glutamine will alter any direct interactions this might make with the channel in the toxin-blocked state. Such interactions are not predictable a priori, since they arise entirely from the specific molecular configuration of the toxin-channel complex. A second type of effect must operate on all positively charged toxins encountering the channel mouth. Nonspecific electrostatic forces concentrate the toxin in the locality of the receptor site according to the magnitude of the local electrostatic potential extending into the aqueous phase. This "concentrative" effect enhances the apparent on-rate of the toxin over what its value would be if the channel mouth were not negatively charged. As ionic strength is raised, these electrostatic forces decrease, and consequently the measured association rate constant falls precipitously.

To test these expectations, we constructed a variant toxin, R19Q/K32Q, with two fewer positive charges than native CTX. We chose Arg19 and Lys32 to neutralize because these positions appear not to make close-range specific interactions with the channel, as evidenced by the absence of altered off-rates in the double mutant (Table I). We chose to study a double mutant in hopes of amplifying any electrostatic effects, which we expected might be quite small; indeed, preliminary data showed that single charge-neutralized mutants behaved similarly to wild-type CTX. This variant, therefore, provides a test of the valence dependence of purely nonspecific electrostatic effects near the channel mouth, unsullied by direct interactions. It fails this test, both quantitatively and qualitatively. Simple electrostatics predicts that the on-rate of the mutant should be lower than that of wild-type CTX and that this lowering should become more pronounced at lowered ionic strength. At 150 mM salt, the on-rate of the double mutant CTX is slightly lower than that of wild-type CTX, but as salt concentration is lowered, the mutant toxin's association rate actually rises slightly above that of CTX. Over the entire range of ionic strength, the on-rate of the reduced-charge toxin roughly parallels that of CTX.

We suggest that this failure arises because CTX is not a point charge and that its charged groups are distributed in space over distances on the order of a Debye length. Such cases are complicated because the electrostatic influence of each charged group depends upon its spatial location on the molecule as well as upon the unknown geometry of the negative charges on the channel protein. The toxin acts as though the charged groups Arg19 and Lys32 are oblivious to the channel's local surface potential, possibly because they are located too far away (25 Å, 3 Debye lengths) from the channel's negative surface charges. This interpretation is buttressed by the fact that "simple" electrostatic behavior has been observed (Green et al., 1987; Green & Andersen, 1991) with blocking of Na^+ channels by monovalent tetrodotoxin and divalent saxitoxin, which are physically much smaller than CTX.

Voltage-Dependent Dissociation and the Mechanism of Toxin Block. With one exception, all the CTX variants studied display voltage-dependent off-rates. Since voltage dependence reflects a strong interaction of K^+ ions within the pore with the CTX molecule on its binding site (MacKinnon & Miller, 1988), we take this as powerful evidence that these mutants bind in a CTX-like configuration. The mutants in which Lys27 is neutralized, however, provide striking exceptions; their dissociation rates are completely independent of transmembrane voltage. This characteristic could be used to argue that K27Q and K27N are so disrupted in structure that they fail to bind to the CTX receptor at all. We resist this interpretation for several reasons. First, like all other CTX derivatives, both K27Q and K27N induce a homogeneous population of nonconducting intervals which are much longer in duration than the channel's spontaneous closed states. Second, the on-rates of these mutant toxins are very close to that of CTX. Third, the K27Q-induced blocked states are competitively inhibited by externally applied TEA, in a fashion quantitatively identical to the effect of this small blocker on wild-type CTX. Finally, 2-D NMR spectra of K27Q show backbone amides virtually identical to those seen in native CTX.

Instead, we propose a more interesting possibility: that the destabilization of CTX by K^+ ions operates by electrostatic repulsion between K^+ in the pore and the ϵ-amino group of Lys27. In this picture, with CTX on its receptor site, Lys27

would be located physically close to the region where the wide channel mouth begins to narrow into the K^+-specific conduction pore. Neutralization of this residue would remove the electrostatic repulsion between pore-associated K^+ and CTX bound at the receptor site and would thus abolish the voltage dependence of the toxin off-rate. Though merely a proposal currently being tested experimentally (Park & Miller, 1992), this model is mechanistically rich in that it motivates further experiments using CTX mutants as probes for the physical structure of the external entryway of this K^+ channel.

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