Differential Roles for Disulfide Bonds in the Structural Integrity and Biological Activity of κ-Bungarotoxin, a Neuronal Nicotinic Acetylcholine Receptor Antagonist†

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ABSTRACT: κ-Bungarotoxin, a κ-neurotoxin derived from the venom of the banded Krait, *Bungarus multicinctus*, is a homodimeric protein composed of subunits of 66 amino acid residues containing five disulfide bonds. κ-Bungarotoxin is a potent, selective, and slowly reversible antagonist of α3β2 neuronal nicotinic acetylcholine receptors. κ-Bungarotoxin is structurally related to the α-neurotoxins, such as α-bungarotoxin derived from the same snake, which are monomeric in solution and which effectively antagonize muscle type receptors (α1β1γδ) and the homopentameric neuronal type receptors (α7, α8, and α9). Like the κ-neurotoxins, the long α-neurotoxins contain the same five conserved disulfide bonds, while the short α-neurotoxins only contain four of the five. Systematic removal of single disulfide bonds in κ-bungarotoxin by site-specific mutagenesis reveals a differential role for each of the disulfide bonds. Removal of either of the two disulfides connecting elements of the carboxy terminal loop of this toxin (Cys 46−Cys 58 and Cys 59−Cys 64) interferes with the ability of the toxin to fold. In contrast, removal of each of the other three disulfides does not interfere with the general folding of the toxin and yields molecules with biological activity. In fact, when either C3−C21 or C14−C42 are removed individually, no loss in biological activity is seen. However, removing both produces a polypeptide chain which fails to fold properly. Removal of the C27−C31 disulfide only reduces the activity of the toxin 46.6-fold. This disulfide may play a role in specific interaction of the toxin with specific neuronal receptors.

The α- and κ-neurotoxins, isolated from the venoms of snakes from the elapid and hydrophid families, are effective antagonists of nicotinic acetylcholine receptors (*1*). While the α-neurotoxins effectively antagonize muscle type receptors (α1β1γδ) and the homopentameric neuronal type receptors (α7, α8, and α9), the κ-neurotoxins are potent, selective antagonists of α3β2 (and to a lesser extent α4β2) neuronal nicotinic receptors (*2*). The binding to these receptors is characterized by very slow kinetic off rates for both classes of toxin. It has also been reported that κ-bungarotoxin is capable of inhibiting receptors containing α2β2, α2β4, α3β4, α4β4, and even α1β1γδ combinations expressed in frog oocytes when the toxin is coapplied with agonist (*3*). In this case, however, the kinetics of inhibition is characterized by very fast off rates. The reason for these differences in kinetic behavior is currently unknown.

In addition to their characteristic interactions with receptors, the α- and κ-neurotoxins also display distinctive structural characteristics. Prominent among these is the observation that the α-neurotoxins are monomeric in solution while the κ-neurotoxins are dimeric in solution. The dimerization of κ-bungarotoxin has been shown to result from specific residues which are conserved in the κ-neurotoxins but not the α-neurotoxins that interact at a dimer interface formed by the two strands of the third loop of each subunit (*4*). The dimeric nature of the κ-neurotoxins may play a role in their binding to receptors and may account at least in part for their different specificity and kinetic characteristics (*5*).

These neurotoxins can also be classified as either short or long neurotoxins based on conserved elements of structure. Short neurotoxins contain 60−62 amino acid residues and four disulfide bridges in common positions. Long neurotoxins usually consist of 66−74 amino acid residues and have a fifth disulfide bond in addition to the four found in short neurotoxins. Short and long neurotoxins are also distinguished by conserved sequence deletions and additions relative to one another (*1*).

Both short and long neurotoxins, including κ-neurotoxins, have been studied extensively by site-directed mutagenesis (*6*−*9*), but there have been few reports regarding the function or role of the conserved disulfide bonds. Do these bonds simply play a structural role in stabilizing the conformation

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of the polypeptide backbone as is usually assumed, or are they required for folding or productive interaction with the receptors? Is there any significance to the presence of a fifth disulfide bond in the long neurotoxins, and what, if any, are its implications to relative toxicity and specific interaction with the receptor binding site? A recent study suggests that the fifth disulfide bond in the long neurotoxins may be required for optimal interaction with the homopentameric α7 neuronal receptor (*10*). Here we describe the effect of the systematic removal by site-directed mutation of each of the five disulfide bonds in κ-bungarotoxin and the implications the results have for both activity and folding of the toxin polypeptide.

MATERIALS AND METHODS

Native and mutant κ-bungarotoxins were expressed in *Pichia pastoris* and isolated as previously described (*11*). Mutagenesis was performed either by the cassette method or by PCR (*12*). All PCR reagents were obtained from Perkin-Elmer. PCR products were purified with a QIAquick PCR purification kit (Qiagen Inc.). Restriction fragments were isolated from agarose gels with a QIAquick gel extraction kit (Qiagen Inc.). Plasmids were isolated with a QIAprep spin miniprep kit (Qiagen Inc.), and all mutations were confirmed by Sanger dideoxy sequencing which was performed with the Sequenase version 2.0 sequencing kit from United States Biochemical.

Toxin proteins were characterized by SDS−polyacrylamide gel electrophoresis and Western blot analysis with rabbit anti-κ-bungarotoxin antisera (*13*). Protein fractionation was performed on a Waters gradient HPLC system as previously described (*11*). Reverse-phase chromatography was performed with solvent A consisting of 0.1% trifluoroacetic acid in water and solvent B consisting of 0.1% trifluoroacetic acid in 90% acetonitrile and 10% water. Ion-exchange chromatography was performed with solvent A consisting of 25 mM potassium phosphate/10% acetonitrile, pH 6.5, and solvent B consisting of 250 mM potassium phosphate/10% acetonitrile, pH 6.5.

Circular dichroism analysis was performed on a Jasco J-600 spectropolarimeter. Secondary structure was qualitatively assessed by a comparison to CD spectra of native venom-derived κ-bungarotoxin. The β-sheet structure of κ-bungarotoxin is characterized by a spectrum that has a strong positive maximum between 190 and 200 nm (*14, 15*). Protein concentration was determined with a Waters Aminoquant amino acid analysis system.

m7G(5′)ppp(5′)G-Capped cRNA was synthesized in vitro from linearized template DNA encoding the α2, α3, α4, β2, and β4 subunits from rat using a mMessage mMachine kit (Ambion). Mature *Xenopus laevis* frogs (Nasco) were anesthetized by submersion in 0.1% 3-aminobenzoic acid ethyl ester, and oocytes were surgically removed. Care and use of *Xenopus* frogs in this study has been approved by the University of Miami Animal Research Committee and meets the guidelines of the NIH. Follicle cells were removed by treatment with collagenase B (Boehringer-Mannheim) for 2 h at room temperature. Oocytes were injected with 20 ng of cRNA in 50 nL of water and were incubated at 19 °C in modified Barth's saline (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.3 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 100 μg/mL gentamicin, 15 mM HEPES, pH 7.6) for 2−7 days. RNA transcripts encoding each subunit were injected at a molar ratio of 1:1. Oocytes were perfused at room temperature (20−25 °C), in a 300-μL chamber with modified Ringers saline (115 mM NaCl, 1.8 mM $CaCl_2$, 2.5 mM KCl, 10 mM HEPES, pH 7.2, 1.0 μM atropine). Perfusion was continuous at a rate of approximately 20 mL/min. Oocytes were exposed to acetylcholine (ACh) for approximately 10 s, using a solenoid valve. κ-Bungarotoxin sensitivity was determined by comparing ACh-induced current responses before and after the oocytes were incubated for 30 min in saline containing various concentrations of κ-bungarotoxin and 100 μg/mL bovine serum albumin. The response to ACh alone, before treatment with κ-bungarotoxin, is taken as the control response. The ACh-induced response after treatment with κ-bungarotoxin is reported as a percent of the control response. Our experimental protocol incorporates a 5-s wash period after κ-bungarotoxin incubation and before measurement of the postincubation ACh response. This wash step eliminates the rapidly reversible blockade of subunit combinations other than α3β2. Thus, our protocol detects only slowly reversible blockade by κ-bungarotoxin. To prevent extensive desensitization, the Ach concentration used to activate each receptor was below the EC_{50}. The EC_{50} for ACh activation is approximately 70 μM for α3β2, 100 μM for α2β2, 200 μM for α4β2, and 210 μM for α3β4 (*16*). ACh concentrations used in our experiments were 1−10 μM for α3β2, 1 μM for α2β2, 100 nM for α4β2, and 30 μM for α3β4. The slowly reversible nature of κ-bungarotoxin blockade of α3β2 allows the postincubation ACh response to be measured without coapplication of toxin. Since toxin and ACh are not in direct competition, the degree of observed block is not dependent on the concentration of ACh, and the ACh concentration used for each receptor need not be equipotent with the ACh concentrations used for other receptors. Current responses were measured under two-electrode voltage-clamp, at a holding potential of −70 mV, using a TEV-200 voltage clamp unit (Dagan Corp.). Micropipets were filled with 3 M KCl and had resistances of 0.5−1.0 MΩ. Current responses were captured, stored, and analyzed on a Macintosh IIci computer using a data acquisition program written with LabVIEW (National Instruments) software (*17*). Inhibition data were fit with Prism 2.0 (Graphpad, Inc.) software using the equation: current = maximum current/[1 + ([antagonist]/IC_{50})n], where n and IC_{50} represent the Hill coefficient and the antagonist concentration producing half-maximal inhibition, respectively.

RESULTS

A series of mutants of κ-bungarotoxin were produced, each having a deletion of one of the five disulfide bonds present in native κ-bungarotoxin. In each case, both cysteine residues that participated in a particular disulfide were changed to alanine residues. The locations of the disulfide bonds in κ-bungarotoxin are shown in Figure 1. Each mutant toxin was expressed in *P. pastoris* and isolated from the culture media. Protein was first purified by reverse-phase HPLC, and all fractions containing material that was immunoreactive with a polyclonal antibody against native κ-bungarotoxin were pooled. These pools were then subjected to ion-exchange chromatography. The resulting elution profiles are shown in Figure 2. Native κ-bungaro-

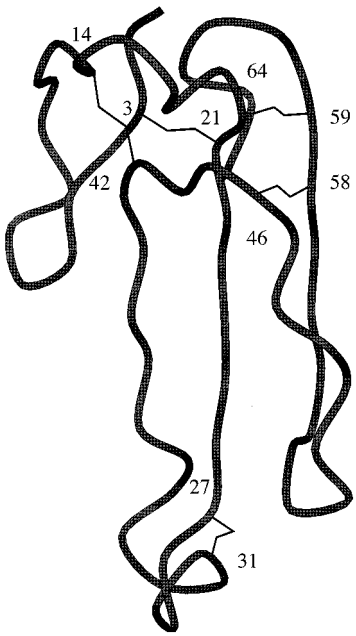

FIGURE 1: Location of disulfide bonds in κ-bungarotoxin. The strand depicts a trace of the α-carbons of one subunit of the κ-bungarotoxin dimer. The three polypeptide loops are referred to as loops 1, 2, and 3 from left to right, respectively. The location of the disulfides are indicated with a solid line joining the appropriate strands, and the numbers indicate the specific cysteine residues forming the disulfide bonds. The dimer is formed by hydrogen bond and van der Waals interaction involving loop 3 of each subunit where the two subunits are related to each other by approximately a 180° rotation in the plane of the paper of the subunit depicted.

toxin elutes as a single peak at approximately 39% solvent B (see Materials and Methods). The C27A/C31A mutant also elutes as essentially a single peak at approximately the same concentration of solvent B. In contrast, the remaining mutants elute as multiple peaks, with C46A/C58A containing a component that elutes in a broad range on either side of 39% B. Fractions were sampled as indicated in Table 1 and analyzed for conformational characteristics by circular dichroism analysis.

Three pools yielded CD spectra with maxima at approximately 198 nm similar to that of native κ-bungarotoxin (Figure 3). These are the single peak from the C27A/C31A mutant and the peaks eluting at approximately 45% B from the C3A/C21A and C14A/C42A mutants. It is noted that the spectra for C14A/C42A and C27A/C31A do not display the usual minima in the region of 215−220 nm. The reason for this is not known at the present time. In addition, the relative intensities of the maxima reflect the relative concentrations of protein used. The remaining pools all produced spectra showing a loss of β-sheet structure which indicates that the mutants affect the ability of the polypeptide to fold properly (*4*).

Toxin pools that displayed CD spectra similar to native κ-bungarotoxin, indicative of β-sheet structure, were compared with wild-type κ-bungarotoxin for the ability to antagonize α3β2 neuronal nicotinic receptors expressed in *Xenopus* oocytes (Figure 4). Figure 4A shows that wild-type κ-bungarotoxin, recombinantly expressed in *P. pastoris*, antagonizes α3β2 neuronal nicotinic receptors with an IC_{50} of 2.30 ± 0.19 nM, very similar to the IC_{50} value (1.16 ± 0.14 nM) obtained with venom-purified κ-bungarotoxin (*18*). At a concentration of 100 nM, recombinant κ-bungarotoxin fails to antagonize the α2β2, α4β2, or α3β4 neuronal nicotinic receptors. Thus, recombinant κ-bungarotoxin displays appropriate potency and specificity. Figure 4B shows that the ability of both the C3A/C21A mutant and the C14A/C42A mutant to antagonize α3β2 receptors is indistinguishable from that of wild-type κ-bungarotoxin. The C27A/C31A mutant toxin was 46.6-fold less potent than wild-type κ-bungarotoxin. IC_{50} values are presented in Table 1.

In light of the results with the C3A/C21A and C14A/C42A mutants, the double-disulfide mutant, C3A/C21A;C14A/C42A, was also constructed. However, unlike the single-disulfide mutants, the double-disulfide mutant failed to give any distinct peaks after ion exchange. Rather, it eluted as a broad band between 30% and 60% solvent B (not shown) similar to that of the second pool of C46A/C58A shown in Figure 2. The CD spectra of this broad peak are shown in Figure 3 and indicate the lack of β-sheet structure. In addition, analysis of this product by Western blot under nonreducing conditions indicated that none of the toxin was present as a monomeric species, suggesting the presence of mixed disulfide aggregates. In contrast, native κ-bungarotoxin, which contains all intermolecular disulfide bonds, runs at the monomeric position on these gels.

DISCUSSION

The mutant κ-bungarotoxins fall into three groups with regard to their biological activity, their ability to fold correctly, and the position of the removed disulfides in the three polypeptide loops of the toxin structure (see Figure 1). The first group is characterized by removal of disulfides formed by cysteine residues in the third loop of the toxin (C46−C58 and C59−C64). Both of these mutants fail to fold properly. The second group consists of one mutant in which the single disulfide internal to the distal end of the second loop (C27−C31) has been removed. This disulfide is often referred to as the fifth disulfide because it is the only one of the five not conserved in the short α-neurotoxins. This mutant maintains the β-sheet structure of the native toxin but shows a substantial loss in activity. The last group is characterized by removal of disulfides that link the first loop to the second loop (C3−C21 and C14−C42). Both mutants in this group maintain the β-sheet structure of the native toxin and retain full biological activity.

Previous work has shown that the integrity of the dimer interface is critical to the ability of the toxin to fold into its active conformation (*4*). Specifically, Phe 49 and Ile 20 were required to form a van der Waals contact across the dimer interface. The crystal structure also indicates that the disulfide bond between Cys 46 and Cys 58 participates in this van der Waals interaction. Removal of this disulfide produces the same result as mutation of either Phe 49 or Ile 20; that is, the toxin fails to fold. However, it is not possible to discern whether this is due to simple disruption of the van der Waals contacts or to a more widespread effect due to generally destabilizing the structure of the third loop. In either case, the effect of removing this disulfide is consistent with the previous finding and serves to support the conclusion that nucleation at the interface appears to be a critical step in the folding of this dimeric molecule. Mutation of the

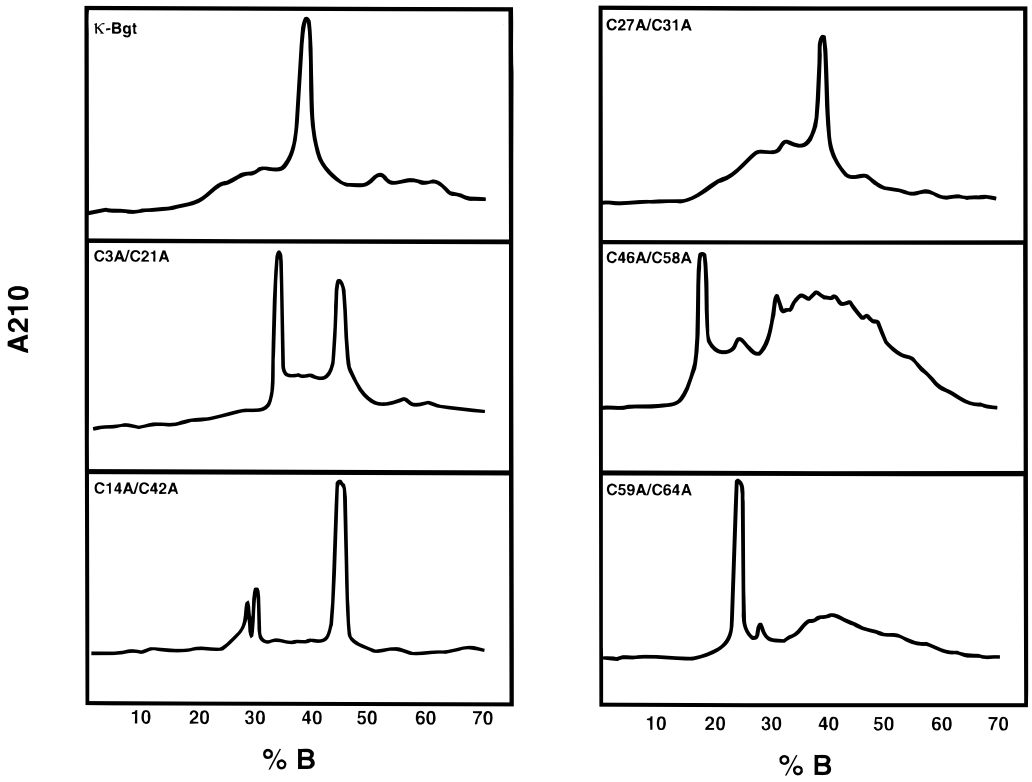

FIGURE 2: HPLC elution profiles of the ion-exchange chromatography of the disulfide mutants. Absorbance at 210 nm is plotted against the percent of solvent B in the gradient: upper left, nonmutated recombinant κ-bungarotoxin; middle left, C3A/C21A mutant; lower left, C14A/C42A mutant; upper right, C27A/C31A mutant; middle right, C46A/C58A mutant; lower right, C59A/C64A mutant.

Table 1: Properties of κ-Bungarotoxin and Disulfide Mutants

κ-Bgt	elution, %	fold	relative yield[a]	activity (IC_{50}, nM, mean ± SD)
venom-purified		β-sheet		1.16 ± 0.14
recombinant	39	β-sheet	100	2.30 ± 0.19
C3A/C21A	34 (peak 1)	random		ND[b]
	45 (peak 2)	β-sheet	60	2.50 ± 0.19
C14A/C42A	30 (peak 1)	random		ND
	45 (peak 2)	β-sheet	100	2.93 ± 0.37
C27A/C31A	39	β-sheet	74	107.1 ± 14.5
C46A/C58A	18 (peak 1)	random		ND
	30−50 (peak 2)	random		ND
C59A/C64A	24	random		ND
C3A/C21A; C14A/C42A	30−60	random		ND

[a] Approximate yield of active species relative to native, recombinant κ-bungarotoxin. [b] ND, not determined.

disulfide between Cys 59 and Cys 64 also appears to disrupt the ability of the toxin to fold. Again, this may also be explained by a previous observation involving mutation at the dimer interface (*4*). The previous studies indicated that the extent of hydrogen bonding across the interface was also critical. For instance, if Arg 54 were removed (ΔR54; *4*), creating a polypeptide shortened by one amino acid, the ability of the toxin to fold productively was also lost. Arg 54 forms a main chain−main chain hydrogen bond across the interface with Thr 60 on the neighboring subunit. Removal of the disulfide formed by Cys 59 and Cys 64 could disrupt this hydrogen bond because of decreased structural rigidity in the region of Thr 60 and produce a similar result.

The remaining mutants all produced toxin polypeptides that folded into β-sheet structure and that exhibited some degree of biological activity. The most seriously compromised in terms of activity was the mutant removing the disulfide formed by C27 and C31 which resulted in a mutant approximately 50-fold less potent than wild type. Servent et al. (*10*) recently suggested that this disulfide was critical to the ability of related toxins to inhibit homomeric α7/$5HT_3$ chimeric neuronal receptors. While long chain α-toxins containing this disulfide bond inhibited muscle type and neuronal receptors to about the same extent, the short chain toxins which do not contain this disulfide, as well as long chain toxins where this disulfide is absent, inhibited the α7/$5HT_3$ receptors between 2 and 4 orders of magnitude less effectively. We find that removing this disulfide from κ-bungarotoxin has a similar, but more modest, effect on the ability of κ-bungarotoxin to antagonize α3β2 neuronal nicotinic receptors. The Servent study also examined the effect of disruption of the disulfide in loop 2 of α-cobratoxin, by selective reduction and chemical modification with 2,2′-dithiopyridine. The resultant thiopyridylated toxin displayed a 2600-fold decrease in affinity for the α7 receptor but no change for the *Torpedo* receptor. However, it is not possible from this study to delineate to what extent this bulky and charged adduct may have affected the toxin's affinity for the neuronal receptor although its affinity is similar to the affinity of those toxins which do not contain this disulfide bond. Our study, where the cysteines are converted to less bulky and uncharged alanine residues, indicates that the effect of losing the fifth disulfide is not nearly as great, at least in the specific case of κ-bungarotoxin interaction with the α3β2 receptor.

Inspection of the structure of long and short toxins indicates that there is indeed a definite structural difference

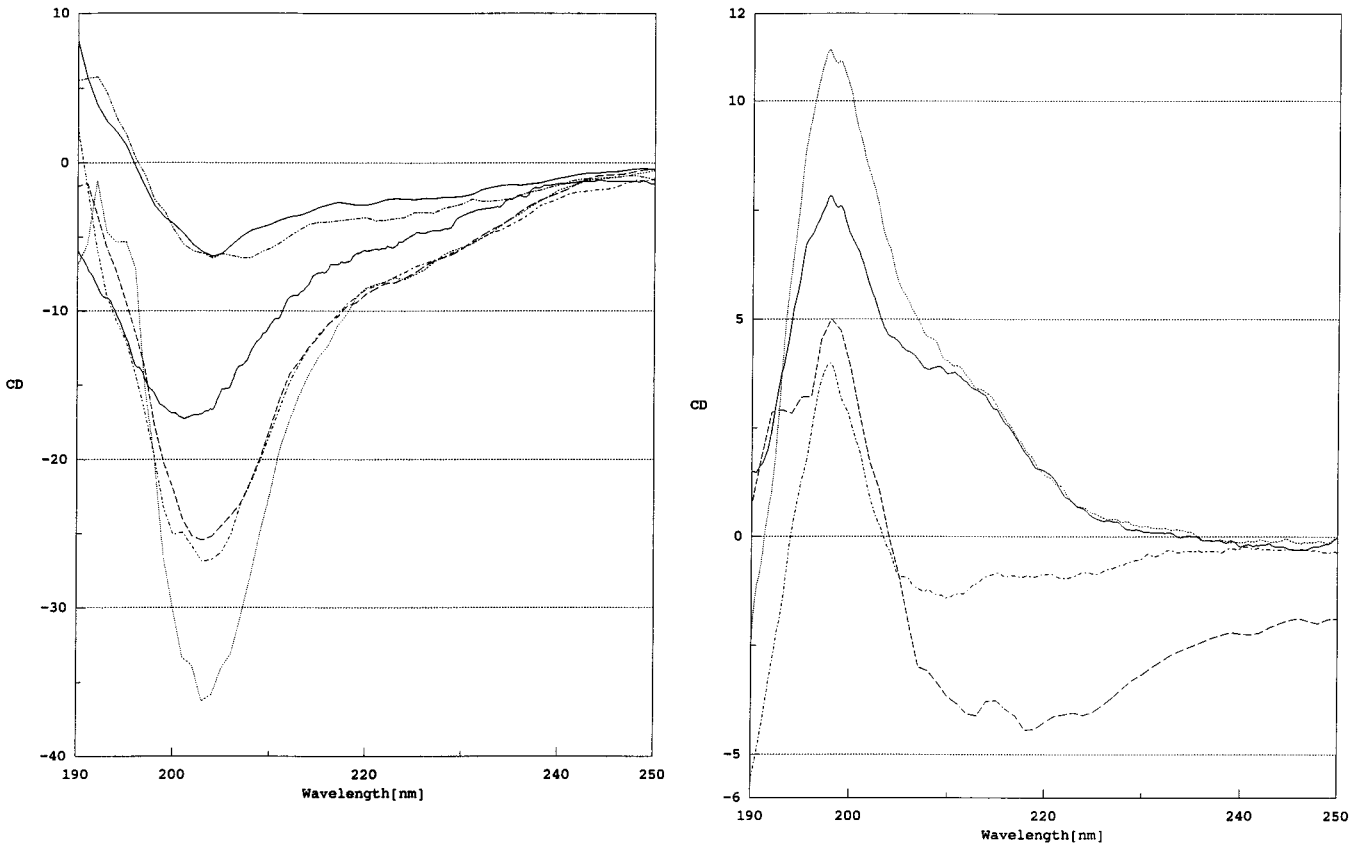

FIGURE 3: Circular dichroism spectra of κ-bungarotoxin mutants. Right: Spectra of unmutated κ-bungarotoxin (− · −), C3A/C21A peak 2 (− − −), C14A/C42A peak 2 (—), and C27A/C31A (···). Left: Spectra of C3A/C21A peak 1 (···), C14A/C42A peak 1 (—, upper), C46A/C58A peak 1 (− · −), C46A/C58A peak 2 (− − −), C59A/C64A (− · · −), and C3A/C21A;C14A/C42A (—, lower).

between them in this area at the end of loop 2. The long toxins contain an additional short stretch of amino acid residues arranged into what looks like a helix turn just before the turn of the loop (Figure 1). This short helical structure appears to be stabilized by this disulfide. The short toxins do not contain this short stretch of helix due to the absence of these additional residues. It was once thought that since the long and short neurotoxins inhibited muscle type receptors, such as that from *Torpedo*, to about the same extent, this "fifth" disulfide was not necessary for activity. The literature is very sparse on the role of this "fifth" disulfide bond, but at least one article (*19*) reports the selective chemical modification of these cysteine residues in α-cobratoxin and concludes that this particular disulfide is not required for binding. The present results and those of Servent indicate that while this may be true for muscle type receptors, it is not true for neuronal receptors and may explain why the venom of snakes that produce these toxins contains both long and short toxins.

In contrast to the previous three disulfide mutants, the C3A/C21A and C14A/C42A mutant activities are indistinguishable from the nonmutated toxin. Both of these disulfide bonds form linkages between a strand of loop 1 and a strand of loop 2. This result suggests that either the first loop participates minimally in the interaction of the toxin with the receptor or this loop is capable of maintaining its structure for the most part in the absence of either of these disulfides. Inspection of the structure of κ-bungarotoxin would suggest that C14A/C42A might be the least critical of the two. In the absence of this disulfide, C3A/C21A is still present to anchor the amino terminus of the protein. In this case, the hydrogen bond interactions between residues of the first and second loop (i.e., between residues Arg 1, Thr 2, Leu 4 and Asp 62, Asn 63, Asn 65 and between Ser 6, Ser 9 and Gln 40) may sufficiently stabilize the structure. On the other hand, with C3A/C21A missing, the amino terminus, which has no other covalent anchor on the amino side of residue 1, may not be sufficiently stabilized by the hydrogen bonds to maintain the proper structure. Nonetheless, its activity is identical to that of the native toxin.

To further explore the role of these two disulfides and loop 1 of the toxin, the double-disulfide mutant, C3A/C21A;C14A/C42A, was constructed. However, it failed to fold into a distinct structure that could be isolated. It is, therefore, not possible to determine any additional insight as to the extent to which the structure of loop 1 contributes to receptor interaction from this construct.

The studies described here have shown that the five disulfide bonds present in κ-bungarotoxin, a snake venom-derived toxin with specificity for certain neuronal nicotinic acetylcholine receptors, display differential roles. Two of the disulfides, C46−C58 and Cys 59−Cys 64, play decidedly structural roles in that their absence disrupts the ability of the toxin polypeptide to fold. This may occur through a destabilization of the dimer interface interaction which has previously been shown to be critical in the folding of this toxin. Two other disulfides, C3−C21 and C14−C42, appear to be unnecessary for full activity when they are individually absent. However, when both are absent in the same

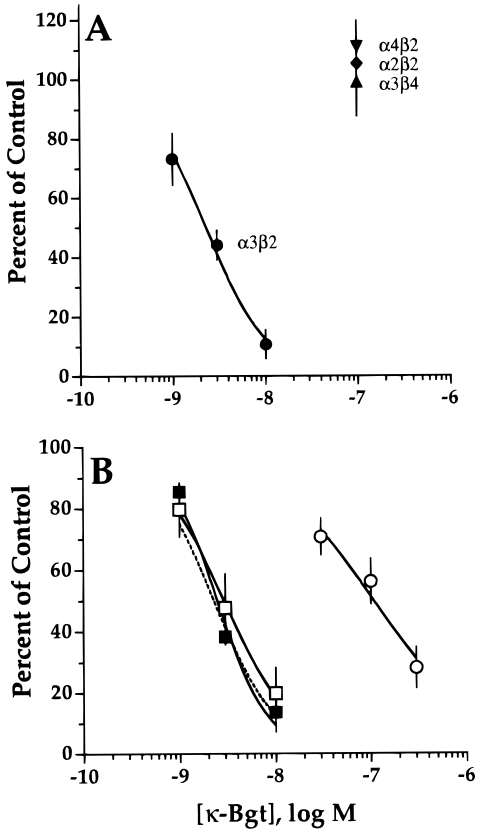

FIGURE 4: Effect of disulfide mutation on κ-bungarotoxin activity. A: κ-Bungarotoxin sensitivity of α3β2 (●), α2β2 (◆), α4β2 (▼) and α3β4 (▲). Current in response to ACh after 30-min incubation with various concentrations of recombinant wild type κ-bungarotoxin is presented as a percentage of the preincubation ACh response (mean ± SD of >3−5 separate oocytes). The line is a fit to a Hill equation (see Materials and Methods). B: Blockade of α3β2 receptors by the C3A/C21A mutant (■), the C14A/C42A mutant (□), and the C27A/C31A mutant (○). Current in response to ACh after 30-min incubation with various concentrations of mutant κ-bungarotoxins is presented as a percentage of the preincubation ACh response (mean ± SD of 3−4 separate oocytes). The lines are fits to a Hill equation (see Materials and Methods). The inhibition curve for wild type κ-bungarotoxin from panel A is shown as a dashed line for reference.

molecule, folding is again compromised. The fifth disulfide, C27−C31, is responsible for an increased affinity for the α3β2 receptor. Its absence decreases the toxin's IC_{50} approximately 50-fold but does not affect the ability of the toxin to fold. The effect of removing this disulfide is consistent with it playing a specific role in the interaction with receptors of neuronal origin as has been proposed (*10*). These studies now set the stage for a more detailed analysis of the role of loop 2 in receptor recognition.

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