Importance of Highly Conserved Anionic Residues and Electrostatic Interactions in the Activity and Structure of the Cardiotonic Polypeptide Anthopleurin B†

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ABSTRACT: Several polypeptide toxins from sea anemones cause delayed inactivation of mammalian voltage-dependent sodium channels, resulting in a positive inotropic effect on the heart. Anthopleurin B (ApB), a toxin produced by the sea anemone *Anthopleura xanthogrammica*, is the most potent of all known anemone toxins. Previous studies in this laboratory have both defined and revealed an important role for the cationic cluster of Arg-12, Arg-14, and Lys-49 in the expression of ApB's biological activity. In the present investigation, we explore the role of all remaining charged residues by producing and characterizing mutants of ApB at Asp-7, Asp-9, Lys-37, His-39, and His-34. Recombinant toxins have been purified to homogeneity and their abilities to enhance veratridine-dependent sodium uptake in cell lines expressing either the neuronal or cardiac isoform of the sodium channel evaluated. Replacement of Asp-7 results in a product that fails to fold, while muteins H39A and H34A have activities very similar or identical to wild-type ApB. In contrast, the D9N and K37A muteins are 7−12-fold less active than wild-type ApB, and truncation of the side chain in D9A results in a further decrease in activity, especially in the cardiac model. We conclude that although a negative charge *per se* is not essential at position 9, the presence of a hydrogen-bond forming side chain is critical both for appropriate folding and for interaction with the sodium channel. Because the K37A and H39A mutant toxins can fold normally, neither Lys-37 nor His-39 seem to participate in an intramolecular salt bridge, in contrast to suggestions arising from NMR studies of ApA and ApB. However, Lys-37 may play a role in channel interaction.

Anthopleurin B, a polypeptide neurotoxin from the sea anemone *Anthopleura xanthogrammica*, has great potential as a heart stimulant, as it interacts with mammalian cardiac sodium channels with high affinity to produce a positive inotropic effect (Renaud et al., 1986). The molecular basis for the cardiostimulatory activity of this toxin needs to be characterized in detail in order to realize its potential as a template for the design of improved cardiotonic drugs.

In previous studies, we have evaluated the contributions of four cationic residues to the activity of this toxin. Analysis of single and double mutants of ApB[1] generated at sites Arg-12, Arg-14, Lys-48, and Lys-49 suggests that these four residues contribute in varying degree to the binding affinity of this toxin for the sodium channel (Gallagher & Blumenthal, 1994; Khera & Blumenthal, 1994; Khera et al., 1995). These studies also reveal a dominant role for Arg-12 in maintaining the high affinity of ApB for neuronal channels.

The roles of Asp-7, Asp-9, and the C-terminal carboxylate group of toxins homologous to ApB have been studied by several different laboratories using chemical modification, but clear-cut results remain elusive. Chemical modification of 2−3 carboxylate groups of ApA (Newcomb et al., 1980) and ASII inactivates these toxins, although surprisingly without affecting the channel binding ability of ASII [*Anemonia sulcata* (Barhanin et al., 1981)]. Gruen and Norton, (1985) found that modification of Asp-7 and Asp-9, but not the C-terminus of ApA, was sufficient to inactivate this toxin as well as severely impairing its native conformation as judged by CD and NMR spectra. In contrast, Mahnir et al. (1990) reported only minor effects on expression of the biological activity of RTX-III (*Radianthus macrodactylus*) upon modification of its carboxylate side chains and no alteration of its secondary structure. In ShI (*Stichodactyla helianthus*), which is structurally related to RTX-III, the anionic triad of Asp-6, Asp-7, and Glu-8 is essential for toxicity but not for maintaining the secondary structure of this toxin.

Thus the question remains as to whether negatively charged residues are important for toxicity, and if so which is required for activity and which for maintaining the active conformation of the toxin? These residues are highly conserved in all known anemone toxins except ASI. Site-directed mutagenesis allows us to assess the functional contributions of the individual aspartate residues, which traditional chemical modification has been unable to establish. In this paper, we report mutations of the highly conserved acidic residues Asp-7 and Asp-9, as well as the conserved Lys-37 and His-39 with which the two aspartate groups have been proposed to interact either ionically or by hydrogen bonding (Pallaghy et al., 1995; Monks et al., 1995). We also included His-34 in this work to explore the hitherto unknown role of its imidazole ring in any molecular interactions in ApB. As with toxin carboxylates, chemical

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[1] [1]Abbreviations: ApA(B), anthopleurin A(B) (neurotoxins A and B from *Anthopleura xanthogrammica*; AsII, neurotoxin II from *Anemonia sulcata*; RTX-III, neurotoxin III from *Radianthus macrodactylus*; ShI, neurotoxin I from *Stichodactyla heliathus.*

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Table 1: Primers used in PCR Mutagenesis[a]

primer	strand	sequence	mutants obtained
PK-8	antisense	5′-gggtct**gggccc**atcagaATTacacagg-3′ *Apa*I	D7N
PK-9	antisense	5′-gggtct**gggccc**ATTagaatcacacagg-3′ *Apa*I	D9N
PK-20	antisense	5′-gggtct**gggccc**aGcagaatcacacagg-3′	D9A
PK-17	antisense	5′-cgga**ccatgg**gcATCacagttgtgcc-3′ *Nco*I	K37D
PK-18	antisense	5′-cgga**ccatgg**gctttacagttC(T/G)(G/C)ccatcc-3′ *Nco*I	H34A/Q
PK-21	antisense	5′-cgga**ccatgg**gcTGCacagttgtgcc-3′ *Nco*I	K37A
PK-10	sense	5′-ctgtaaagcc(CG)(AC)Gggtccg-3′	H39A/Q
PK-11	antisense	5′-cggaccC(TG)(GC)ggctttacag-3′	H39A/Q

[a] Restriction sites used in cloning mutant-containing PCR products are shown in boldface, and the sites of mutation given in uppercase.

modification has given a mixed message as regards the functional role of histidine residues in anemone toxins (Newcomb et al., 1980; Barhanin et al., 1981).

Our results clearly indicate different functions for the two ApB carboxylates, with Asp-7 being more important for folding and Asp-9 playing a role in both channel affinity and toxin folding. In contrast, Lys-37 is involved only in toxin binding, and neither of the two histidine residues plays a significant role in either folding or affinity for the channel. The implications of these results for previously published models of the contributions made by these residues to the structure and function of *Anthopleura* neurotoxins is discussed.

EXPERIMENTAL PROCEDURES

Cell Cultures. Murine neuroblastoma cells (N1E-115) were kindly made available by Dr. Marshall Nirenberg (National Heart, Lung, and Blood Institute, NIH). The cell line RT4-B, known to express predominantly the cardiac/denervated skeletal muscle sodium channel, was generously provided by Dr. Laurie Donahue (Health Sciences Center, Texas Tech University). High glucose growth medium containing 10% fetal calf serum and 110 units/mL each of penicillin and streptomycin was used to grow cells to confluency. In order to enhance adherence of N1E-115 cells to culture plates for sodium flux assays, cells were differentiated for 2 days prior to assay in medium containing 1.5% fetal calf serum and 1.5% dimethyl sulfoxide.

Mutant Constructions. All mutants were constructed by the PCR method using synthetic oligonucleotides containing the desired mutation. The primers used for mutagenesis are shown in Table 1, and the expression vector pKB-13 (Dias-Kadambi et al., 1996) encoding the ApB gene fused to the 3′-end of gene 9 of bacteriophage T7 was used as a template in all cases. Gel-purified *Eco*RI/*Apa*I or *Eco*RI/*Sst*I digests of PCR products were inserted into pKB-13 following cleavage with the appropriate restriction enzymes. Because the H39A mutation destroys the unique *Nco*I restriction site, this mutein was constructed by overlap extension (Ho et al., 1989; Horton et al., 1989). Sequences of all mutants were confirmed by double-stranded dideoxy chain termination (Sanger et al., 1980).

Expression and Isolation of ApB Mutants. Mutant proteins were expressed and purified as described previously (Gallagher & Blumenthal, 1992). Following anion-exchange chromatography on DE52, disulfide bonds were formed using redox couples of glutathione, and the fusion proteins were then hydrolyzed with staphylococcal protease. Final purification was carried out by reverse-phase HPLC.

Analytical Methods. Amino acid analyses of wild-type and mutant proteins were performed by standard procedures. All samples were hydrolyzed in the presence of 6 N HCl at 110 °C for 22 h. Dried hydrolysates were derivatized with phenyl isothiocyanate and their compositions analyzed by HPLC on a Pico Tag column.

The influence of each mutation on secondary structure was estimated by measuring far-UV circular dichroism spectra on a Jasco J-710 spectropolarimeter. The conformational stabilities of all mutant proteins were tested by thermal denaturation experiments carried out in the presence of 1.5 M guanidine hydrochloride. In both cases, secondary structure contents were calculated by comparing spectral data to a least squares fit of a reference spectrum containing the known structures of myoglobin, egg white lysozyme, ribonuclease A, papain, cytochrome C, hemoglobin, α-chymotrypsin, trypsin, and horse liver alcohol dehydrogenase.

Functional Characterization of Wild-Type and Mutant ApB Proteins. To determine the biological activities of the toxin variants, veratridine-dependent $^{22}Na^+$ uptake by cultured cells was measured as described previously (Gallagher & Blumenthal, 1992). In brief, cells were preincubated at 37 °C with sodium-free binding medium containing 20 μM veratridine and increasing concentrations of wild-type or mutant toxins. The absence of sodium in the preincubation medium prevents dissipation of the membrane potential due to channel activation during toxin binding. Initial rates of sodium uptake were then measured by incubating the cells in an isotonic uptake medium containing the same concentrations of toxins and 10 mM $^{22}NaCl$. Uptake rates were corrected for baseline sodium uptake due to 20 μM veratridine alone and the experimental data fitted to a single hyperbolic function by the method of Cleland (1979) to obtain the kinetic constants $K_{0.5}$ and V_{max}.

RESULTS

Choice of Mutations. Two kinds of mutations were generated for each aspartic acid residue. The first replaces the carboxylate with an amide to determine the importance of a negative charge in the side chain. Second, since asparagine is capable of hydrogen bonding similar to aspartic acid, replacement by alanine, which removes both the charge and the hydrogen bonding potential, was also studied. In addition, Lys-37, His-34, and His-39 were mutated to K37A, H34A, and H39A, respectively. Finally, to test the predicted possibility of a salt bridge between Asp-7 and Lys-37, reversal of charges was also attempted by constructing the double mutant D7K-K37D.

Table 2: Amino Acid Compositions of Anthopleura Toxins

amino acid	residues per mol[a]					
	ApB	D9N	D9A	H34A	H39A	K37A
Asx	4.7 (5)	4.8 (5)	4.1 (4)	5.0 (5)	5.6 (5)	5.1 (5)
Glx	0.6 (0)	0.7 (0)	0.8 (0)	0.7 (0)	1.2 (0)	1.3 (0)
Cys	4.0 (6)	nd[c]	nd	nd	nd	nd
Ser	3.6 (4)	3.5 (4)	3.7 (4)	3.7 (4)	3.8 (4)	3.7 (4)
Gly	8.0 (8)	7.7 (8)	8.0 (8)	7.9 (8)	8.2 (8)	8.0 (8)
His	1.7 (2)	1.9 (2)	2.0 (2)	1.0 (1)	1.0 (1)	1.9 (2)
Thr	1.3 (1)	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)	0.8 (1)
Ala	1.4 (1)	1.0 (1)	2.2 (2)	2.3 (2)	1.9 (2)	2.2 (2)
Arg	2.1 (2)	1.9 (2)	2.0 (2)	1.9 (2)	2.2 (2)	2.0 (2)
Pro	5.9 (6)	6.1 (6)	5.1 (6)	5.3 (6)	5.3 (6)	6.6 (6)
Tyr	0.9 (1)	1.0 (1)	1.0 (1)	1.0 (1)	1.2 (1)	1.0 (1)
Val	1.4 (1)	1.0 (1)	1.0 (1)	1.0 (1)	1.2 (1)	1.0 (1)
Met	0.1 (0)	0.1 (0)	0.1 (0)	0.0 (0)	0.0 (0)	0.0 (0)
Ile	2.3 (2)	2.1 (2)	2.3 (2)	2.1 (2)	1.5 (2)	2.3 (2)
Leu	3.2 (3)	3.0 (3)	2.9 (3)	3.0 (3)	2.7 (3)	2.8 (3)
Phe	1.3 (1)	1.0 (1)	1.0 (1)	1.1 (1)	1.0 (1)	1.1 (1)
Lys	2.9 (3)	3.1 (3)	2.9 (3)	3.2 (3)	2.9 (3)	1.8 (2)
Trp[b]	(3)	(3)	(3)	(3)	(3)	(3)
MW[d]	5272.9 (5275)[e]	5272.5 (5274)	5233.3 (5231)	5205.1 (5209)	5206.2 (5209)	5214.5 (5218)

[a] Numbers in parentheses are based on the predicted amino acid sequence of either wild-type or mutated forms of ApB. [b] Determined spectrophotometrically. [c] Not determined. [d,e] Molecular weights were measured by matrix-assisted laser desorption time of flight mass spectrometry of HPLC-purified toxins and are compared to molecular weights calculated from the amino acid sequence.

Table 3: Secondary Structures of Anthopleura Toxins

toxin form	% α-helix	% β-sheet
ApB	0.8	52.3
D9N	0.0	54.4
D9A	0.0	52.5
H34A	0.0	55.3
H39A	0.0	55.5
K37A	0.0	50.9

Reoxidation, Cleavage, and Purification of Fusion Protein. Mutant ApB proteins were expressed after induction of *Escherichia coli* strain BL21(DE3) harboring plasmid encoding the desired mutant ApB gene. All mutant fusion proteins were expressed at levels comparable to those of the wild-type protein. Cleavage of the reoxidized wild-type fusion protein and the D9N, K37A, H34A, and H39A containing fusion proteins resulted in similar yields of toxin following HPLC purification, whereas the D7A and D7K-K37D fusion proteins failed to yield an intact toxin, and only very small amounts of the D7N mutein could be isolated. Our inability to isolate either D7A or D7K-K37D proteins, and the minimal yield obtained for D7N, strongly suggests an important role for Asp-7 in folding. Yields of the D9N mutant are similar to wild-type products, whereas the final yields of D9A are again quite low, indicating that the presence of a hydrogen bond forming residue and not an acidic residue at position 9 is also important in maintaining the native conformation of ApB.

Structural Characterization of Mutant Proteins. The amino acid composition of each purified protein agrees well with that of the wild-type protein and confirms in each case the presence of the desired substitution (Table 2). To confirm that the mutations generated do not perturb the secondary structures of mutant proteins, far-UV circular dichroism spectra of all proteins were examined over the range of 190−250 nm. All spectra are indistinguishable from those of the wild-type protein. Secondary structure estimations derived from the spectral data (Table 3) indicate that, like wild-type ApB, all muteins exist in a predominantly β-sheet conformation with contents ranging from 51 to 56%. Structural stabilities of these mutant toxins were also compared with the wild-type form. Thermal denaturation profiles carried out in the presence of 1.5 M guanidine hydrochloride demonstrate that neither the secondary structure nor the thermal stability of ApB is affected by any of the point mutations studied at positions 9, 34, 37, and 39 (data not shown).

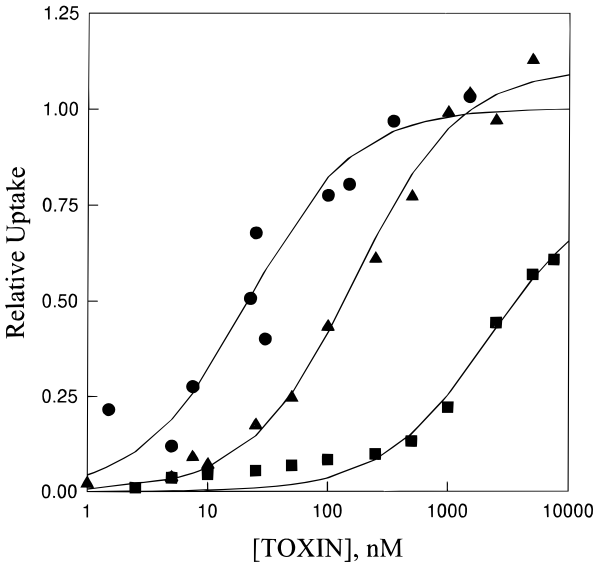

FIGURE 1: Effects of ApB on veratridine-dependent sodium uptake by N1E-115 cells. The synergistic effect of wild-type ApB (●), and the ApB mutants D9N (▲) and D9A (■) on uptake induced by 20 μM veratridine. Uptake rates were determined as described in Experimental Procedures, corrected for the basal uptake due to veratridine and normalized to that of wild-type ApB. The solid lines represent theoretical curves and the points are the experimental data.

Functional Characterization. All mutants were characterized by measuring their ability to synergistically enhance veratridine-dependent sodium uptake by cultured cells expressing either the neuronal (N1E-115) or cardiac (RT4-B) isoform of the sodium channel. In our ion flux assays, the $K_{0.5}$ value reflects the apparent binding affinity of toxin for the sodium channel, while V_{max} represents the ability of toxin to maintain the channel in its open conformation. Dose−response curves for wild-type ApB and D9N and D9A mutants for neuronal channels are compared in Figure 1, and those for cardiac channels are compared in Figure 2. The kinetic constants $K_{0.5}$ and V_{max} determined for these and the remaining muteins tested are summarized in Table 4.

The $K_{0.5}$ values for the H39A mutant in both cell lines are very similar to that of the wild-type toxin as are those for H34A (Table 4). Because these mutations remove all possible contributions made by the imidazole ring, neither histidine is essential for interaction with the sodium channel. Moreover, our ability to produce both the H34A and H39A muteins in normal yields argues strongly that neither imidazole side chain is significantly involved in toxin folding.

Replacement of the remaining residues targeted has more substantial effects on folding and/or binding affinity. Substitution of Lys-37 by alanine causes a 7-fold decrease in apparent binding affinity of ApB for neuronal sodium channels and 11-fold reduced affinity for cardiac channels. Because the K37A toxin appears to fold normally (Table 3), these data suggest Lys-37 is not directly involved in any

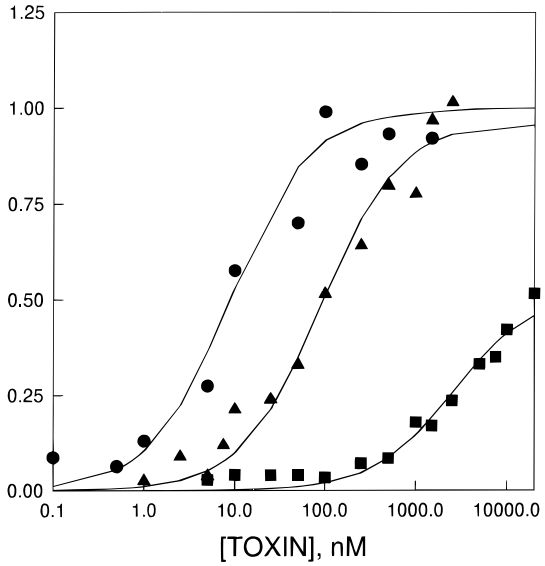

FIGURE 2: Effects of ApB on veratridine-dependent sodium uptake by RT4-B cells. The synergistic effect of wild-type ApB (●), and the ApB mutants D9N (▲) and D9A (■) on uptake induced by 20 μM veratridine. The uptake rates shown have been corrected for the basal uptake due to veratridine and normalized to that of wild-type ApB.

Table 4: Effects of ApB Mutations on $K_{0.5}$ and V_{max}

	$K_{0.5}$ (nM)		V_{max} (% wild type)	
isoform	neuronal	cardiac	neuronal	cardiac
ApB	22 ± 3	9 ± 3	1.0	1.0
D9N	165 ± 14	88 ± 8	1.1 ± 0.03	1.0 ± 0.02
D9A	2147 ± 173	2554 ± 217	0.8 ± 0.03	0.5 ± 0.02
K37A	146 ± 12	100 ± 7	1.0 ± 0.02	1.1 ± 0.02
H34A	41 ± 3	14 ± 4	1.1 ± 0.02	1.0 ± 0.07
H39A	23 ± 3	15 ± 2	1.0 ± 0.03	1.1 ± 0.03

electrostatic interactions important to stabilization of the protein structure. Instead, the approximately 10-fold decrease seen in binding affinity is consistent with the Lys-37 side chain contributing about 1 kcal/mol to stabilization of the toxin−channel complex.

Similarly, introduction of an amide group at Asp-9 (D9N) leads to 8- and 10-fold decreased affinity for neuronal and cardiac channels, respectively. When Asp-9 is replaced with an alanine, however, a dramatic decrease in toxin activity results, accompanied by substantially diminished yields. The $K_{0.5}$ of the D9A mutant is 2.15 μM in N1E-115 cells, almost 100 times lower than that of wild-type ApB. The $K_{0.5}$ of D9A in the cardiac system is similar to that in N1E-115s, reflecting a 284-fold reduction in apparent binding affinity. As the saturation point of sodium uptake was not attained even at 20 μM, our $K_{0.5}$ value for the D9A is almost certainly an underestimate.

These data indicate an important functional role for the Asp-9 carboxylate, as abolition of its negative charge with retention of hydrogen-bonding potential causes an approximate 10-fold decrease in binding affinity. The additional loss of activity seen upon side chain truncation (D9A) may indicate that a hydrogen bond at this position is able to partially compensate for loss of the anionic function, but because there is clearly an additional effect on toxin folding, it is difficult to draw unambiguous conclusions on this issue from our data. Finally, as the very limited amount of D7N obtained shows only a 4−6-fold lower binding affinity for neuronal and cardiac channels, respectively, it is unlikely that Asp-7 is functionally important. Most likely, it plays primarily a structural role in ApB.

DISCUSSION

Chemical modification studies carried out by several different laboratories have suggested that acidic residues at positions 7 and/or 9 of the sea anemone toxins ApA, ASII, and ShI are essential for the expression of toxin activity (Newcomb et al., 1980; Barhanin et al., 1981; Pennington et al., 1990), but the wide variations in extent of inactivation have left this conclusion open to question. In the reports cited above, toxicity was abolished upon carboxylate blockade, but Mahnir et al. (1990) reported only 2−6-fold reduced activity upon chemical modification of one or two carboxylates of RTX-III without any alteration in its secondary structure. On the other hand, when Gruen and Norton (1985) examined the CD and NMR spectra of a glycine ethyl ester-modified derivative of ApA, the conformation of the modified toxin was significantly altered, suggesting that Asp-7, Asp-9, and the C-terminal carboxylate are essential for maintaining the secondary structure of ApA. Analogous chemical modification analyses of ASII and ApA, both close homologues of ApB, could not ascertain whether one or both aspartate residues were essential for toxin structure and/or function. The inability of these earlier investigators to reach a clear-cut conclusion as to the roles of these residues is due in part to their failure to succeed in modifying only one residue at a time and to then study its effect on toxin structure and function. We have solved this problem of targeting single sites by employing site-directed mutagenesis to replace any single residue with an amino acid of choice and to then study its contribution to toxicity and/or conformation.

In this paper we have examined the roles of Asp-7 and Asp-9 of ApB, and also the contributions of Lys-37 and His-39, which are located close to both of the aspartate residues and on the same surface of the molecule (Pallaghy et al., 1995; Monks et al., 1995). We have also investigated the previously uncharacterized role of the imidazole ring of His-34 to the expression of toxin activity. Our results show that Asp-7 is an important structural residue, since upon its replacement by asparagine, we are unable to refold ApB, although expression levels of the fusion protein are comparable to those seen for wild type. Characterization by ion flux assay of the very small amount of the D7N mutant we were able to generate and purify, while incomplete, indicates that Asp-7 is not an important functional residue, since the apparent binding affinity of D7N toxin to neuronal and cardiac channels is only 4−6-fold lower than that of wild-type ApB.

Our results further indicate that Asp-9 is an important functional residue, as its replacement by asparagine leads to a 10-fold loss in apparent binding affinity, while substitution with an apolar alanine results in a more dramatic decrease. From these data we conclude that, minimally, a hydrogen-bond-forming residue is essential at position 9 for maintaining ApB's channel binding affinity. Truncation of the carboxylate side chain of Asp-9 (D9A) not only considerably inactivates the toxin but also results in much lower yields of the folded polypeptide. Hence, it seems a hydrogen-bonding residue may also be important at position 9 for facilitating toxin folding. These results present quite a contrast to those of Barhanin et al. (1981), who suggested that chemical modification of the three carboxylates of ASII

completely destroyed toxin activity while leaving channel binding capability intact. This result is difficult to reconcile with most models of anemone toxin action, as well as with our data, which clearly show a loss of apparent binding affinity (4−8-fold) to the neuronal channel upon replacement of *either* Asp-7 or -9 with asparagine. Because both of our mutants also retain significant activity in the ion flux assay, it is unlikely that their conclusion regarding abolition of ASII activity upon complete carboxylate modification can be correct. It is most likely that the discrepancy in activity between these two studies is related either to introduction of a bulky blocking group, to side reactions during carbodiimide treatment, or to simultaneous modification of the C-terminal carboxylate in the earlier study. Our structural model of ApB (Khera et al., 1995), and the known structures of other anemone toxins, suggests that the C-terminal carboxylate is situated near the cationic cluster which we have previously implicated as being important in toxicity. This could account for differences in toxicity between the two studies, as reflected by ion flux activities. However, it cannot explain the failure of Barhanin et al. to observe any change in toxin binding affinity, and the basis for this discrepancy remains unresolved.

Chemical modification of both His-34 and His-39 of ApA by Newcomb et al. (1980) did not inactivate this toxin, whereas Barhanin et al. (1981) reported a significant loss of toxicity and binding affinity upon modification of the two histidines of ASII. Our analysis of the H34A and H39A toxins indicates that mutein H39A is as active as wild-type ApB while H34A shows only a minimal loss of biological activity. Hence, the imidazole rings of His-34 and His-39 are not important for the function of ApB.

Because the K37A mutation causes a 7-fold reduction in binding affinity for neuronal and an 11-fold reduction for cardiac sodium channels as compared with the wild-type ApB, Lys-37 plays a significant role in ApB activity. That we can make this mutant in normal quantities suggests that Lys-37 does not take part in stabilizing the structure of ApB through an electrostatic interaction with Asp-7, as proposed by Pallaghy et al. (1995).

Both Asp-7 and Asp-9 are located in or near the conformationally unstructured loop common to anemone toxins (Norton et al., 1980; Widmer et al., 1989; Monks et al., 1995; Pallaghy et al., 1995). NMR analyses of ApA and ASII indicate that one of these two aspartates has an abnormally low pK_a and that protonation of this residue at low pH alters protein structure. These observations have been interpreted as indicating that a carboxylate side chain is involved in stabilizing electrostatic interactions similar to a salt bridge (Norton & Norton, 1979; & Norton et al., 1980). However, because these two carboxylates are very close to each other, it is not clear which one has the low pK_a. Pallaghy et al. (1995) speculate that in ApA it is Asp-7, since its side chain is closer than that of Asp-9 to the ammonium group of Lys-37 and thus more apt to participate in a salt bridge. These investigators further suggest that Asp-7, Lys-37, and His-39 are close enough to form a cluster, such that Asp-7 might also interact with His-39. In ApB, however, it is the imidazolium ring of His-39, rather than the ϵ-amino group of Lys-37, which is closer to the aspartate side chains and thus better able to participate in ionic interactions (Monks et al., 1995). Our characterization of ApB mutants at these sites suggests that neither histidine *per se* plays an essential role in such interactions, since we see no indication that either the H34A or H39A toxins is compromised either in its ability to fold or its overall conformational stability. Of the sites proposed by Norton and co-workers to participate in essential stabilizing interactions in the *Anthopleura* toxins, we see evidence for an important role only for Asp-7. None of the potentially cationic groups appear to play a crucial role in ApB folding, although Lys-37 clearly contributes to overall binding affinity, and because of their proximity it is possible that, upon removal of any single cationic group in this region, others are able to compensate for its absence. These findings will be useful for the potential use of ApB in the design of new cardiotonic drugs.

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