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An Essential Binding Surface for ShK Toxin Interaction with Rat Brain Potassium Channels†

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ABSTRACT: An "Ala scan" analysis of ShK toxin, a 35-residue basic peptide possessing three disulfide bonds, identifies seven side chains which influence binding to brain delayed rectifier potassium channels. Additional analogs were synthesized and tested to further decipher the roles of these residues, particularly Tyr23. The inhibitory effects of these analogs on ^{125}I-labeled dendrotoxin binding to rat brain membranes showed that replacement of Tyr23 with Ala drastically lowered the affinity of the toxin for the Kv1.2 channels. Ala substitution of Phe27 reduced potency more than 15-fold. Monosubstituted Ala analogs for Ile7, Ser20, or Lys30 each displayed 5-fold reductions in potency. Thus, aromaticity at position 23 is important for effective delayed rectifier brain K channel binding. In contrast, the aromatic residue at position 27 was not critical, since cyclohexylalanine substitution increased affinity. The solution structure of ShK toxin clusters Ile7, Arg11, Ser20, Lys22, Tyr23, and Phe27 in close proximity, forming the potassium channel binding surface of the toxin. We propose an essential binding surface on the toxin in which Lys22 and Tyr23 are major contributors, through ionic and aromatic (hydrophobic) interactions, with the potassium channel.

Potassium channels regulate numerous biological processes. The tissue-specific expression of certain voltage-gated (Kv) delayed rectifier type channels makes them potential targets for the therapeutic treatment of certain diseases. For instance, the homomeric Kv1.3 expressed in T-lymphocytes modulates proliferation of these cells and thus is a target for development of new anti-inflammatory drugs (Chandy et al., 1984; Leonard et al., 1992). Detailed analysis of the interactions of scorpion toxins with this channel has provided a model for the active surface of these toxins (Aiyar et al., 1995).

Recently, a new class of potassium channel blocking toxins was discovered in three species of sea anemones (Karlsson et al., 1991; Aneiros et al., 1993; Castaneda et al., 1995; Schweitz et al., 1995). The three toxins are basic molecules which range in size from 35 to 37 residues, contain three disulfide bonds (Pohl et al., 1995), and display much sequence homology. We were able to synthesize one of these toxins using solid-phase methods in order to obtain sufficient amounts for NMR structure determination and for pharmacological investigations (Pennington et al., 1995). We found that ShK toxin binds both to rat brain membranes containing predominantly Kv1.2 channels and to Jurkat T-lymphocytes possessing Kv1.3 channels. Using monosubstituted analogs, we found that Lys22 was essential for effective rat brain K channel binding but not binding to the lymphocyte Kv1.3 channel. Toxin binding to lymphocyte Kv1.3 channels was also adversely affected by increasing

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side chain bulk (i.e., Arg, Nle, homocitrulline) at position 22 (Pennington et al., 1996). In contrast, Arg11 was critical for lymphocyte binding (Pennington et al., 1996), but its replacement with Gln did not greatly affect binding to rat brain Kv channels.

In this report, we have exploited solid-phase methods to efficiently prepare synthetic analogs of ShK in order to complete an "Ala scan" of the entire toxin, excluding the six half-cysteinyl residues and one Gly residue. In addition, we prepared several additional monosubstituted analogs to further evaluate other sites previously identified as important. Finally, several deletion and multiply substituted analogs were prepared with an ultimate goal of reducing the size of the peptide toxin while maintaining its binding domain.

EXPERIMENTAL PROCEDURES

Natural Toxins. Dendrotoxin I (DTX) isolated from *Dendroaspis polylepis* was graciously provided by Dr. E. Karlsson, Biomedical Center, University of Uppsala, Uppsala, Sweden. Wild-type ShK toxin and radioiodinated dendrotoxin I (^{125}I-DTX) were prepared according to previously described methods (Pennington et al., 1995). All other reagents were of the finest grade commercially available.

Synthesis of ShK Toxin Analogs. Fmoc amino acids (Bachem Feinchemikalien, Bubendorf, Switzerland) included Ala, Arg(Pmc), Asn(Trt), Asp(OtBu), cyclohexylalanine (Cha), Cys(Trt), Gln(Trt), Glu(OtBu), Gly, His(Trt), Ile, Leu, Lys(Boc), Met, Phe, Pro, Ser(tBu), Thr(tBu), and Tyr(tBu). Stepwise assembly was carried out starting with 10 g of Fmoc-Cys(Trt)-resin (0.65 mmol/g) on a Labortec SP640 peptide synthesizer. Aliquots were removed throughout the synthesis of the entire 35-residue sequence to facilitate analog synthesis by eliminating redundant steps. At this point, resin aliquots were removed and placed on an Applied Biosystems 431A peptide synthesizer at the 0.25 mmol scale, and the remaining amino acid sequence incorporating the substitution was assembled according to the previously described procedure (Pennington et al., 1996). The K30A, T31A, and T34A analogs were synthesized entirely on an ABI 431A according to the procedure used to prepare wild-type ShK toxin (Pennington et al., 1995). Following final removal of the Fmoc group, each of the peptides was cleaved from the resin and simultaneously deprotected with reagent K (King et al., 1990) for 2 h at room temperature. The free peptide was then filtered to remove the spent resin beads and precipitated with ice-cold diethyl ether, collected on a fine filter by suction, washed with ice-cold ether, and finally extracted with 20% AcOH in H_2O. Oxidative folding of the disulfide bonds and its subsequent purification were as previously described (Pennington et al., 1995) with the following exception: oxidative folding of the K30A analog required the presence of 1.5 mM reduced glutathione and 0.75 mM oxidized glutathione. Each analog was purified using preparative RP-HPLC as described previously (Pennington et al., 1995, 1996). HPLC-pure fractions (purity >95%) were pooled and lyophilized. Synthetic yields ranged from 8% (K30A) to 22% (S2A and T34A). Structures and the purity of all the analogs were confirmed by HPLC, circular dichroism spectroscopy, amino acid and ESI-MS analysis.

Binding Assays. The procedures for measuring displacement of ^{125}I-DTX binding to rat brain membranes were previously described (Pennington et al., 1995). In brief, ShK toxin and its analogs were incubated with rat brain membranes (0.2 mg of protein) and 1 nM ^{125}I-DTX (60 Ci/mmol) for 1 h at room temperature. The incubation was carried out in a buffer containing 150 mM NaCl, 30 mM Tris-HCl, and 1 mg/mL BSA, pH 7.0, in a total volume of 0.25 mL. Nonspecific binding was measured with 1 μM cold DTX. The measurements were performed in triplicate. The standard error of mean measurement was less than 6%.

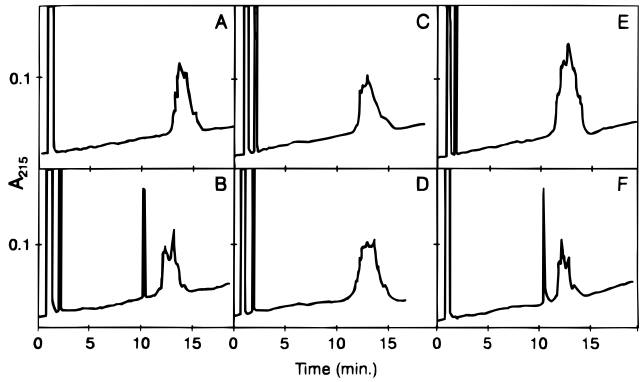

FIGURE 1: Analysis of oxidative folding of ShK toxin analogs by RP-HPLC. Each trace represents an injection of 50 μg of total peptide onto an ODS column (Vydac 0.46 × 25 cm) developed with a linear gradient of aqueous acetonitrile gradient from 5% to 45% in 20 min with a flow rate of 1.5 mL/min. Panels: (A) K30A after folding for 36 h in the presence of air, (B) K30A after folding for 18 h in the presence of 1 mM reduced and oxidized glutathione, (C) D5A after folding for 36 h in the presence of 1 mM reduced and oxidized glutathione, (D) D5E after folding for 36 h in the presence of 1 mM reduced and oxidized glutathione, (E) H19A after folding for 36 h in the presence of 1 mM reduced and oxidized glutathione, and (F) H19K after oxidative folding for 18 h in the presence of air.

RESULTS AND DISCUSSION

Air oxidation of the linear precursors afforded peptides having proper disulfide pairings as the major product. In the case of K30A, we found that this analog required the addition of glutathione to afford a major product (Figure 1A,B, peak eluting at 10.61 min). In our previous report, we speculated that this residue is critical for normal toxin folding (Pennington et al., 1996). The solution structure of the toxin places the ϵ-amino group of Lys30 in close proximity to the Asp5 carboxyl (Tudor et al., 1996). Tudor and Norton (personal communication) have found that certain Lys30 and Asp5 protons titrate together, which is also consistent with Lys30 being involved in an ionic interaction which may affect toxin folding. Our several attempts at folding Asp5 analogs D5A and D5E (Figure 1C,D) also failed. The failure of D5E to fold properly suggests that the distance separating the two ionizable groups is also quite critical.

Our Ala scan data also implicate His19 as important for folding. Repeated folding attempts with H19A, including addition of glutathione, failed to yield a major product (Figure 1E). However, a correctly folded H19K analog was isolated by HPLC (Figure 1F, peak eluting at 11.04 min). The His residue occurs at the end of the first helical segment in ShK toxin (Tudor et al., 1996); a polar/basic residue at position 19 may be essential for ShK folding, perhaps by stabilizing the helical dipole.

The single Pro residue occurring at position 8 in ShK toxin resides between an extended peptide bond conformation and

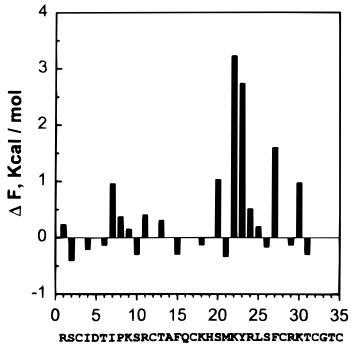

FIGURE 2: Free energy difference of binding for ShK toxin analog as determined by displacement of ^{125}I-labeled dendrotoxin binding to rat brain membranes of ShK analogs. The free energy difference of binding was calculated as $\Delta F = RT \ln(IC_{50}$ of analog/IC_{50} of WT toxin), where $R = 1.987$ cal/mol and $T = 295$ K. Amino acid residues substituted with Ala are shown in single-letter code. The analogs with a single substitution for Cys (C) or Gly (G) were not synthesized in the present study as these residues were expected to be important for proper folding of the toxin. Data for Ala substitutions at positions Arg1, Phe15, Lys18, Lys22, and Arg24 were previously reported (Pennington et al., 1996) but here have been converted to free energy values and included to make the Ala scan analysis complete.

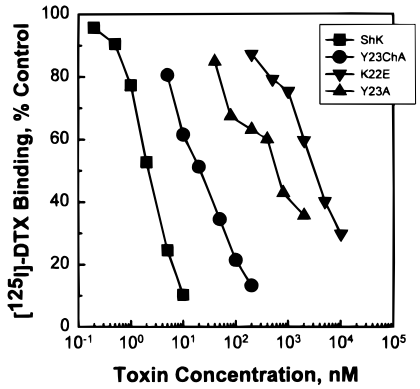

FIGURE 3: Displacement of ^{125}I-DTX (1 nM) specific binding to rat brain membranes by ShK toxin and several analogs.

a possible 3_{10} helix (Tudor et al., 1996). Alanine substitution at this position did not affect folding (as studied by HPLC and CD analysis) and only slightly affected K channel binding. The other homologous sea anemone potassium channel toxins lack Pro.

The CD spectra (data not shown) of all the folded analogs were virtually identical to that of ShK toxin (Kem et al., 1996), providing evidence that the conformation of each analog was similar to that of native ShK. Wild-type ShK toxin has a characteristic CD spectrum indicative of a protein containing approximately 30% α-helix (Kem et al., 1996).

The binding affinities of ShK toxin and its analogs for rat brain K channels, which are predominantly Kv1.2 channels, were determined by inhibition of specific ^{125}I-labeled dendrotoxin binding to rat brain membranes. Differences between the free energies of binding of the Ala scan analogs relative to ShK toxin are plotted in Figure 2. (A positive ΔF value means toxin affinity was less than that of the wild-type toxin sequence.) Replacement of Tyr23 with Ala (Y23A) resulted in the largest reduction of affinity (Figure 3) and more than a 2.8 kcal/mol increase in the relative free energy of binding (Figure 2). Replacement of Phe27 with Ala also lowered the affinity by nearly 1.6 kcal/mol. Three monosubstituted analogs, I7A, S20A, and K30A, displayed moderately reduced affinity amounting to an increase of approximately 1 kcal/mol. Most other substitutions resulted in free energy differences which were very small (<0.5 kcal/mol), indicating binding similar to that of the wild-type toxin.

Table 1: Affinity of ShK Toxin Analogs for Displacing ^{125}I-Labeled Dendrotoxin from Rat Brain Membranes

toxin analog	equipotent molar ratio (analog IC_{50}/ShK IC_{50})	free energy difference of binding, ΔF (kcal/mol)
N^{α}-Ac	1.0	0.0
des-R1-S2[a]	2.5	0.54
R11E	30.8	2.01
Q16E	2.8	0.60
H19K	2.6	0.56
K22E	726.0	3.86
Y23Cha	8.8	1.27
F27Cha	0.7	−0.21
A4 + A15[b]	0.5	−0.41
A15 + A25[b]	2.1	0.43
A4 + A15 + A25[b]	0.2	−0.94

[a] Analog where N-terminal Arg and Ser were deleted. [b] Multiply substituted analog where each relative substitution site is referred to by number in the ShK sequence.

Several analogs actually showed increased binding affinity relative to wild-type ShK toxin. As shown in Figure 2, F15A, K18A, M21A, S26A, R29A, and T31A displayed increased affinities, reflected in a negative value for the relative free energy of binding values. We suggest that reducing steric bulk at these sites allows easier access of the toxin to the K channel vestibule.

Additional monosubstituted analogs were prepared to evaluate our hypothesis that Lys22 is crucial for rat brain Kv1.2 binding. We speculated that Lys22 might interact with an anionic residue, like Asp402, in the Kv1.3 model (Guy & Durell, 1994; Aiyar et al., 1995). Replacement of its basic side chain with an anionic side chain was expected to inhibit insertion of this portion of the toxin into the lumen of the K channel pore. As shown in Table 1 and Figure 3, K22E displayed a greatly reduced affinity for the rat brain K channel. The R11E analog also displayed a 30-fold decrease in affinity, which implicates this side chain as part of the Kv1.2 pharmacophore. Our present data indicate that the Lys22 side chain is the most critical basic residue in the toxin for interaction with rat brain Kv1.2 channels.

The importance of the final two basic groups in ShK, the α-amino group and the His19 imidazole ring, were respectively tested by preparing the N^{α}-Ac derivative and the H19K analog. We also prepared the truncated des-R1-S2 peptide in an attempt to shrink the size of the molecule in a manner similar to that in a recent report on atrial natriuretic factor (Li et al., 1995). As shown in Table 1, neither acetylation nor truncation of the amino terminus significantly affected ShK toxin binding. The H19K analog displayed a slightly reduced IC_{50} ratio, which supports our hypothesis of a structural role for this residue.

The potential importance of the planar aromatic side chains at positions 23 and 27 was also assessed with several analogs of ShK toxin. Saturation of the aromatic ring results in a nonplanar hydrophobic cyclohexylalanine (Cha) side chain. Substitution of Cha for Tyr23 (Y23Cha) resulted in more than an 8-fold reduction in potency (Table 1 and Figure 3). For future photoaffinity labeling experiments, we prepared Y23Bpa (*p*-benzoyl-Phe). Increasing the bulkiness of the side chain in Y23Bpa only slightly decreased the binding

affinity (Kem, Pennington, and Mahnir, unpublished results). Thus, analogs at position 23 show that a planar aromatic side chain is important. In contrast, replacement of Phe27 with Cha slightly increased toxin affinity; we conclude that hydrophobicity is important here, but a planar aromatic side chain is not necessary.

Lastly, Table 1 lists several additional analogs in which two to three hydrophobic residues were simultaneously substituted with Ala. According to our model, which docks the ShK toxin into a model of the lumen of the Kv1.3 channel, reducing the bulk of these side chains should decrease the dimensions of the channel-binding surface of the toxin and thereby facilitate insertion of the toxin into the channel vestibule (Chandy, Norton, Pennington, and Kem, in preparation). As shown in Table 1, each of the multiply substituted analogs, A4 + A15 + A25 and A4 + A15, is more potent than the wild-type toxin. These data are consistent with our current model orienting the Lys22 amino group into the K channel pore lumen (Pennington et al., 1996). The disubstituted analog A15 + A25 was slightly less potent than the wild-type toxin, which may indicate that reducing the size/bulk of the side chain has a greater effect at position 4 relative to position 15 or 25.

Our new data provide strong evidence that the Tyr23 residue present in all three sea anemone K channel toxins isolated to date is an important residue for binding to at least one of the Shaker-type K channels. We previously reported that the phenolic hydroxyl was not essential, as Y23F binding affinity was essentially identical with that of the wild-type toxin (Pennington et al., 1996). Nevertheless, an aromatic moiety is clearly important at this position, since the Y23A and Y23Cha analogs respectively displayed >100-fold and >8-fold lower affinities for rat brain channels relative to ShK toxin (Figure 3).

The binding surface of ShK toxin appears to involve the side chains of at least six amino acid residues. Most of these residues are located in the region from 20 to 30 of the toxin sequence. As shown in Figure 4, most of these residues are clustered together on the surface of the solution structure of ShK toxin. The two critical residues (Lys22 and Tyr23) are proximal to each other with the lysine side chain most exposed. Most of the other residues (Ile7, Arg11, Ser20, and Phe27) which also appear to influence ShK toxin binding are clustered around these two residues. Only Lys30 is not found within this binding surface. This residue appears to be involved in a salt bridge with Asp5, and thus an Ala substitution may slightly alter the binding properties due some minor conformational change. It is also possible that this site may interact with one of the walls of the K channel outer vestibule, similar to the perimeter basic residues in the charybdotoxin homologs (Aiyar et al., 1995).

Thus, as with charybdotoxin (Park & Miller, 1992; Stampe et al., 1994), ShK toxin binding to the delayed rectifier Kv1 type K channels apparently is dependent upon only a few side chains on the toxin surface. In charybdotoxin, Lys27 is most important and apparently penetrates into the pore of the channel. Lys27 occurs in the middle of a β-sheet in charybdotoxin (Bontems et al., 1992). In the recently reported solution structure of ShK toxin, the critical Lys22 and Tyr23 side chains reside in an α-helical segment of the toxin (Tudor et al., 1996). Thus, ShK toxin appears to use a totally novel scaffold to orient a very similar binding surface or pharmacophore into the outer vestibule of the K channel.

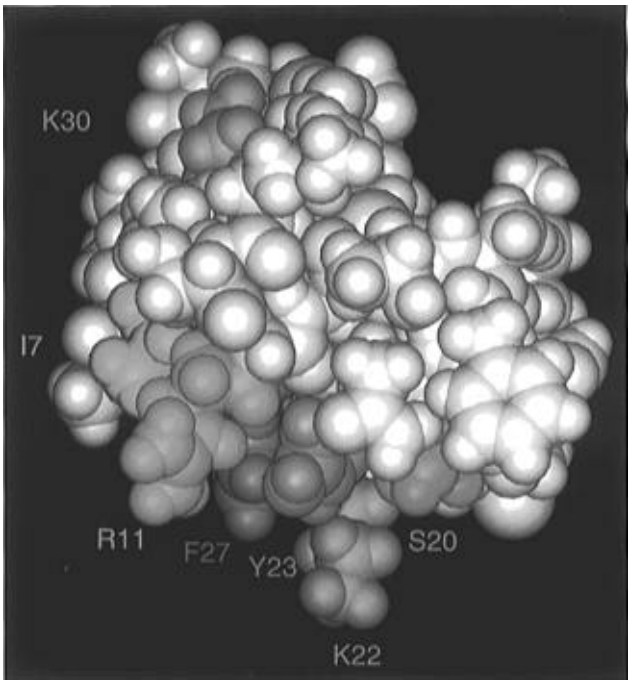

FIGURE 4: CPK surface diagram of ShK toxin (Tudor et al., 1996) with the side chains of Ile7 (light green), Arg11 (cyan), Ser20 (dark green), Lys22 (blue), Tyr23 (magenta), Phe27 (orange), and Lys30 (blue) highlighted. [CPK surface diagram of ShK toxin was generated from the 2D ^{1}H NMR spectra as described in Tudor et al., (1996).] Coordinates of ShK toxin have been deposited in the Protein Data Bank (Accession Number 1ROO).

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