The Signature Sequence of Voltage-gated Potassium Channels Projects into the External Vestibule*

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A highly conserved motif, GYG(D/E)GD, contributes to the formation of the ion selectivity filter in voltage-gated K⁺ channels and is thought to interact with the scorpion toxin residue, Lys27. By probing the pore of the Kv1.3 channel with synthetic kaliotoxin-Lys27 mutants, each containing a non-natural lysine analog of a different length, and using mutant cycle analysis, we determined the spatial locations of Tyr400 and Asp402 in the GYG(D/E)GD motif, relative to His404 located at the base of the outer vestibule. Our data indicate that the terminal amines of the shorter Lys27 analogs lie close to His404 and to Asp402, while Lys27 itself interacts with Tyr400. Based on these data, we developed a molecular model of this region of the channel. The junction between the outer vestibule and the pore is defined by a ring (–8–9 Å diameter) formed from alternating Asp402 and His404 residues. Tyr400 lies 4–6 Å deeper into the pore, and its interaction with kaliotoxin-Lys27 is in competition with K⁺ ions. Studies with dimeric Kv1.3 constructs suggest that two Tyr400 residues in the tetramer are sufficient to bind K⁺ ions. Thus, at least part of the K⁺ channel signature sequence extends into a shallow trough at the center of a wide external vestibule.

Voltage-gated K⁺ channels play diverse physiological roles in both excitable and non-excitable tissues and are attractive therapeutic targets for various diseases (1). These channels contain six putative transmembrane segments (S1 to S6) and a membrane-associated loop between S5 and S6 (P-region). The ion conduction pathway, bounded at its external and internal entrances by wide vestibules, is formed in large part by the P-region and the C-terminal half of S6 (2–8). The P-region of all cloned voltage-gated K⁺ channels contains a highly conserved G(Y/F)GD motif (1), which is thought to constitute an essential part of the K⁺ ion selectivity filter (9–12).

The external vestibule is the receptor site for several scorpion peptide toxins that are potent blockers of K⁺ currents (7, 12–16). In an earlier study, we used four structurally related scorpion toxins as molecular probes to map the topology of this region in Kv1.3 (7), a voltage-gated K⁺ channel that plays a vital role in modulating lymphocyte activation (17–19). This was achieved by determining the three-dimensional structures of the toxins and by identifying multiple pairs of interacting toxin and Kv1.3 residues (7). Knowing the disposition of these interacting toxin residues from their NMR structures, we were able to deduce the architecture of a 30-Å-wide and ~8-Å-deep vestibule at the outer entrance to the Kv1.3 pore (7). The scorpion toxins interact with all four subunits in the Kv1.3 tetramer, and the conserved toxin residue, Lys27, protrudes into the pore, possibly interacting with residues in the signature sequence (7). By replacing Lys27 in kaliotoxin (KTX)1 with positively charged non-natural lysine analogs of varying chain lengths, we were able to show that the terminal amine at toxin position 27, positioned at distances anywhere from 2.2 to 7.7 Å from Cc, can interact suitably with the pore (7).

In the present study, we have used this series of KTX-Lys27 mutants as a caliper to estimate the vertical distance between His404 in the external vestibule, and Tyr400 and Asp402, two residues contained within the signature sequence of Kv1.3. Using site-specific mutagenesis coupled with thermodynamic mutant cycle analysis (14, 20), we assessed the interaction strength and estimated the distances between the terminal amines in each of these Lys27 analogs and each of the three channel residues. Our data suggest that Asp402 and Tyr400 tie in a shallow depression at the center of a wide saucer-shaped outer vestibule. Based on these experimental data, we have developed a modified molecular model of the outer mouth of the Kv1.3 pore.

EXPERIMENTAL PROCEDURES

KTX Mutants—The five KTX-Lys27 mutants have been described previously (7). Four of these contain positively charged non-natural lysine analogs of varying side chain lengths (DAP, 2.5 Å from C; DAB, 3.8 Å; Orn, 5.0 Å; ThLys, 7.7 Å); the neutral non-natural amino acid, Nle27 (5.0 Å) replaced Lys27 (6.3 Å) in the fifth mutant. Another KTX mutant used in this study, R24D, was also described earlier (7). Three batches of synthetic KTX (7) were obtained from Dr. James Boyd, Pfizer Central Research, Groton, CT. The first batch was used for all the His404 mutant experiments; the second and third, slightly more potent batches were used for studies on the WT-D402N and WT-Y404V dimers and for the protonation experiments.

Kv1.3 Mutants—WT Kv1.3 and six Kv1.3-H404 mutants (H404N, H404S, H404T, H404A, H404L, and H404V), have been described previously (7). A WT-D402N dimer was generated by joining WT and D402N monomers, in frame, via a glutamine (Gln10) linker using a unique BamHI site. The linker was engineered into subunit 1 (WT Kv1.3) using the polymerase chain reaction. The two domains of the WT-Y404V dimer were linked by a serologically detectable MRGSH6 epitope via a unique BamHI restriction site.

Biophysical Characterization—Kv1.3 cRNA (WT, mutant or dimer) were transcribed in vitro using a kit purchased from Ambion Inc. (Austin, TX) and injected into oocytes (Xenopus laevis) purchased from Nasco, Fort Atkinson, WI as described (21). K⁺ currents were measured at room temperature using the two-electrode voltage-clamp tech-

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and their mutants was calculated using the formula, $P_{425}$, $L_{427}$, $T_{449}$, $G_{452}$, $P_{453}$, and $W_{454}$ in the model of the action does not exclude the possibility of close proximity, since residues are against a single mutant toxin containing the neutral residue, Nle.

Mutational Mutant Cycle Analysis—Mutant cycle analysis is a simple and powerful tool to study the strength of interaction between any pair of residues in proteins (14, 20, 22). For each mutant cycle, we measured the potency ($K_r$ values) of KTX and each of its mutants on WT-Kv1.3 and each of the channel mutants. The change in coupling energy, $\Delta G$ (20), for a given pair of interacting toxin-channel residues and their mutants was calculated using the formula, $\Delta G = kT\Delta n$, where $\Omega$ (14) is a dimensionless value given by the formula: $\Omega = K_r(\text{WT toxin-WT Kv1.3}) \times K_r(\text{mut toxin-mut Kv1.3}) / K_r(\text{mut Kv1.3-WT toxin}) \times K_r(\text{WT Kv1.3-mut toxin})$. An $\Omega$ value of 1 indicates no interaction, while values deviating from 1 suggest stronger energetic contacts (14).

The distance between each pair of residues was estimated using the criteria of Schreiber and Fersht (20) who demonstrated a strong correlation between $\Delta G$ values obtained by mutant cycle analysis and inter-residue distances obtained from the crystal structures of Barnase and Barstar. They showed that a $\Delta G$ value of $\pm 0.5$ kcal mol$^{-1}$ corresponded to an inter-residue distance of $\pm 5$ Å (20 error), and this value was concluded as $\Delta G$ increased. Note that although high $\Delta G$ values indicate tight interactions, failure to detect an energetically coupled interaction does not exclude the possibility of close proximity, since residues that are physically close may be energetically “silent.” This is especially true when random scanning mutagenesis is used to identify coupling (12, 22).

In our experiments we have assumed that KTX and all its mutants sit in the vestibule with a similar geometry, with the side chains of residues at position 27 interacting with the pore in their fully extended conformation. For the purpose of comparison, the toxins containing positively charged residues at position 27 (DAP, DAB, Orn, Lys, ThLys) were treated as “WT” in the mutant cycle analyses and evaluated against a single mutant toxin containing the neutral residue, Nle. Modeling the Kv1.3 Pore—To create a model of the Kv1.3 pore, Phe$^{255}$, Lys$^{447}$, Thr$^{449}$, Gly$^{452}$, Phe$^{453}$, and Tyr$^{454}$ in the model of the Shaker channel (3) were mutated to Gly$^{306}$, Asn$^{362}$, His$^{404}$, Thr$^{407}$, Ile$^{408}$, and Gly$^{409}$, respectively. Assuming 4-fold symmetry, the distances we experimentally estimated between residues Gly$^{306}$, Asp$^{356}$, His$^{404}$, Asp$^{405}$, and Tyr$^{404}$ (Ref. 7 and this paper) were included as constraints during optimization of the model (for distances and to correct for any bad Van der Waals contacts) with SYBYL 6.2 (Tripos Inc., St. Louis, MO).

RESULTS AND DISCUSSION

Terminal Amines of Shorter KTX-Lys27 Analogs Are Close to His$^{404}$ in Kv1.3—His$^{404}$, located at the entrance to the pore (7), forms the external binding site for tetraethylammonium (23, 24). Using mutant cycle analyses, we estimated the strength of interaction between His$^{404}$ and each of terminal amines at Kv1.3 position 27. Examples of such experiments for a short (DAP$^{27}$) and long (Lys$^{27}$) residue are shown in Fig. 1A ($K_r$ values in Table I). Replacing His$^{404}$ with the hydrophobic valine (H404V), strongly perturbed the interaction of the short analog with the pore ($\Omega = 150$), but not that of the longer analog ($\Omega = 3.4$; Fig. 1A). These results suggest that His$^{404}$ and KTX-DAP lie close to one another.

A more extensive mutagenesis study supported this conclusion (Fig. 1B, Table I). Mutant cycle experiments involving various hydrophobic substitutions at channel position 404 produced lower $\Omega$ values for the toxins containing the shorter lysine analogs (DAP$^{27}$, DAB$^{27}$, Orn$^{27}$) than did polar replacements (Asn, Ser, Thr); none of these His$^{404}$ mutations significantly altered $\Omega$ values for the longer analogs, Lys$^{27}$ and Lys$^{37}$ (Fig. 1B). These results suggest that His$^{404}$ might hydrogen-bond with the terminal amines of the shorter KTX-Lys$^{27}$ analogs, since this interaction is maintained by polar, but not hydrophobic, substitutions.

If the short analogs are close to His$^{404}$ as these data suggest, protonation of His$^{404}$, brought about by changing the external pH from 6.8 to 6.0 (7), would be expected to reduce their potency via electrostatic repulsion of the terminal amine at position 27; such an effect should be less pronounced for the longer toxin-lysine 27 analogs that do not interact energetically with His$^{404}$. To test this idea, we performed mutant cycle experiments, measuring the $K_r$ values for each of the KTX-Lys$^{27}$ pairs: DAP = 19, DAB = 22, Orn = 3, Lys = 2, ThLys = 1. $\Omega$ values for the Kv1.3-Tyr$^{404}$-KTX-Lys$^{27}$ pairs: DAP = 2, DAB = 3, Orn = 2, Lys = 13, ThLys = 2.

ThLys$^{27}$ (Fig. 1B). These results suggest that His$^{404}$ might hydrogen-bond with the terminal amines of the shorter KTX-Lys$^{37}$ analogs, since this interaction is maintained by polar, but not hydrophobic, substitutions.

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The $\Delta G$ values for each of the Kv1.3-H404V-KTX-DAP27Nle and Kv1.3-H404V-KTX-K27Nle $\Omega$ values are shown for each cycle. In these cycles, the positively charged analogs (KTX-DAP$^{27}$ and KTX-Lys$^{27}$) are compared against a single neutral toxin mutant, KTX-Nle$^{27}$, B, three-dimensional bar graph showing $\Omega$ values for various KTX-Lys$^{27}$-channel-His$^{404}$ interactions. C, distance from Kv1.3-H404V-KTX-K27Nle; to Tyr$^{404}$/Asp$^{405}$/His$^{404}$. Horizontal axis, length of side chain at KTX position 27 from C$_{\alpha}$ (A). Vertical axis, coupling energy. The dotted line represents the 0.6 kcal mol$^{-1}$ cutoff (20). $\Omega$ values for the Kv1.3-Asp$^{402}$, KTX-Lys$^{27}$ pairs: DAP = 19, DAB = 22, Orn = 3, Lys = 2, ThLys = 1. $\Omega$ values for the Kv1.3-Tyr$^{404}$-KTX-Lys$^{27}$ pairs: DAP = 2, DAB = 3, Orn = 2, Lys = 13, ThLys = 2.

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Asp⁴⁰² in Kv1.3, has long been an attractive candidate for a salt-bridge interaction with Lys²⁷. Unfortunately, it has not been possible to test this idea, since mutations of Asp⁴⁰² result in non-functional channels. We circumvented this problem by using a dimeric construct consisting of one wild-type and one mutant (D⁴⁰²N) subunit, which yielded a functional channel in which the overall negative charge at position 402 is halved.

The WT-D⁴⁰²N dimer (Fig. 2A, center) inactivates more rapidly than the WT Kv1.3 channel (Fig. 2A, left). Dose-response curves demonstrate that all five toxins containing positively charged residues at position 27 blocked the WT Kv1.3 channel with almost equal potency (Fig. 2B, left; Table I), while the WT-D⁴⁰²N dimer was significantly less sensitive to DAP²⁷ and DAB²⁷ than to the longer analogs (Fig. 2B, middle; Table I). The neutral KTX-Nle²⁷ toxin mutant blocked WT Kv1.3 and the dimer with substantially lower potency than the positively charged KTX-Lys²⁷ forms (Table I). The ΔΔG values obtained from mutant cycle experiments (Fig. 1C) represent strong interactions between Asp⁴⁰² and the shorter KTX-Lys²⁷ analogs, indicating that Asp⁴⁰², like His⁴⁰⁴, must lie within 5 Å of the terminal amines in the shorter KTX-Lys²⁷ analogs.

### Table I

| Lys²⁷ Analogs | DAP | DAB | Orn | Lys | Thlys | Nle |
|---------------|-----|-----|-----|-----|-------|-----|
| WT Kv1.3      | 0.24(0.13) | 0.17(0.14) | 0.14(0.05) | 0.10(0.1) | 0.82(0.66) | 52(27) |
| H⁴⁰⁴A         | 25(3.5) | 33(4.2) | 27(9.8) | 0.72(0.11) | 2.80(1.0) | 160(46) |
| H⁴⁰⁴L         | 520(198) | 390(82) | 440(56) | 28(2) | 49(14.2) | 610(37) |
| H⁴⁰⁴V         | 36(8.5) | 36(11) | 35(11) | 0.30(0.15) | 1.1(0.8) | 51(22) |
| H⁴⁰⁴T         | 11(3.5) | 13(3.9) | 14(8.2) | 0.33(0.26) | 0.46(0.17) | 36(7.8) |
| H⁴⁰⁴S         | 8.0(6.2) | 6.5(4.8) | 39(23) | 0.85(0.07) | 2.0(0.63) | 130(36) |
| H⁴⁰⁴N         | 31.7(12) | 44.6(4) | 19.2(1) | 38.2(14) | 18.8(4.8) | 1100(127) |
| H⁴⁰⁴Y         | 0.27(0.14) | 0.41(0.35) | 0.48(0.45) | 0.03(0.03) | 0.07(0.01) | 0.17(0.02) |
| H⁴⁰⁴, pH 6.8   | 0.65(0.20) | 1.3(0.50) | 0.34(0.04) | 1.4(0.27) | 0.82(0.27) | 155(56) |
| H⁴⁰⁴, pH 6     | 54(16.6) | 40(16.8) | 9.9(4.1) | 15(5.5) | 27(6.0) | 210(45) |
| WT Kv1.3      | 0.13(0.1) | 0.17(0.12) | 0.13(0.05) | 0.04(0.02) | 0.33(0.15) | 22(5) |
| WT-D⁴⁰²N      | 16(5.6) | 18(2.5) | 2.0(0.8) | 0.38(0.07) | 1.7(0.3) | 140(119) |
| WT-Y⁴⁰⁰V      | 1.5(0.42) | 2.5(0.72) | 3.8(0.05) | 2.5(0.61) | 3.6(1.35) | 110(32) |

*Three batches of toxins were used in the study, each with slightly differing potency.*

### Footnotes

1 Values in parentheses represent standard deviation. n = 3–8 per experiment.

**Fig. 2.** A, currents in Xenopus oocytes expressing WT-Kv1.3 (left), WT-D⁴⁰²N dimer (middle), and WT-Y⁴⁰⁰V dimer (right). B, dose-response curves for WT-Kv1.3 (left), WT-D⁴⁰²N dimer (middle), and WT-Y⁴⁰⁰V dimer (right). C, mutant cycles showing the effect of changing external K⁺ ion concentration on the KTX-Lys²⁷–Kv1.3-Tyr⁴⁰⁴ interaction (left) and the KTX-Nle²⁷–Kv1.3-Tyr⁴⁰⁴ (right) pairs.

Earlier work on the Shaker channel suggested that Lys²⁷ in agitoxin-2 interacted with a residue at or close to a K⁺ ion binding site within the pore (12); occupation of this site by a K⁺ ion destabilized the toxin interaction with the channel via electrostatic repulsion of Lys²⁷ (12). Similarly, K⁺ competitively inhibited the interaction between Lys²⁷ in charbydotoxin and residues in the pore of the “ maxi” calcium-activated K⁺ channel (25, 26). Since Kv1.3-Tyr⁴⁰⁴ is close to KTX-Lys²⁷, it is a good candidate for such a K⁺ ion binding site. We examined the effect of changing the external K⁺ concentration from 2 to 100 mM on the blocking potency of KTX-Lys²⁷ or KTX-Nle²⁷ on either the WT channel or the WT-Y⁴⁰⁰V dimer. If Tyr⁴⁰⁴ is a K⁺ binding site, its interaction with KTX-Lys²⁷, but not KTX-Nle²⁷, should be disrupted as the K⁺ ion concentration in the pore increases. Results with the WT-Y⁴⁰⁰V dimer will depend on whether K⁺ binds to all four residues in the tetramer or whether two will suffice. For example, if two tyrosines are sufficient for binding K⁺, then increasing the K⁺ concentration in the pore should competitively inhibit KTX-Lys²⁷ binding to the dimer and consequently produce a high ΔΔG value by mutant cycle analysis. Alternatively, if four tyrosines participate in K⁺ binding, then halving the number (as in our dimer), would diminish the channel’s affinity for K⁺ ions and reduce the ability of external K⁺ to compete with KTX-Lys²⁷ for Tyr⁴⁰⁴. The mutant cycle experiments presented in Fig. 2C favor the first alternative.

As shown in Fig. 2C (left), increasing external K⁺ concentration reduced by 6-fold, the potency of KTX-Lys²⁷ on the WT Kv1.3 channel, and this effect was even more pronounced (38-
firm the feasibility of the experimentally determined interactions and to predict new ones. Docking was achieved by guiding the KTX-Lys$^{27}$ residue into the center of the pore and then rotating the toxin around the central axis of the pore until Arg$^{400}$ was aligned with Asp$^{396}$. This docking configuration (Fig. 3A) does not violate any of the previously reported toxin-channel interactions, including Arg$^{400}$-Asp$^{396}$, Asn$^{402}$-Asp$^{396}$, Ser$^{1}$-His$^{2}$, Phe$^{42}$-His$^{404}$, Met$^{29}$-His$^{404}$, and Thr$^{9}$-His$^{404}$ (7, 12, 14, 16).

In summary, using a series of KTX-Lys$^{27}$ mutants as molecular yardsticks, we have determined the spatial location of two residues in the K$^{+}$ channel signature motif (Asp$^{402}$ and Tyr$^{400}$), relative to a third residue, His$^{404}$, in the external vestibule of Kv1.3 (7). A molecular model based on these and earlier (7, 12, 14, 16) experimental data reveals the existence of a 30-Å wide outer vestibule, at the center of which lies a ring, consisting of four alternating Asp$^{402}$ residues and His$^{404}$ residues, which defines the boundary between the external vestibule and the pore. Positioned 4–6 Å deeper into the pore is Tyr$^{400}$, which interacts with KTX-Lys$^{27}$ and binds K$^{+}$ ions, possibly via cation/pi-electron interactions (27). Our model has considerable experimental support, both from our results (Ref. 7 and this paper) and those of others (12, 14, 16). It provides a structural basis for understanding the biophysical properties involving the external vestibule and pore, including ion permeation and C-type inactivation. The model might also facilitate the design of novel non-peptide blockers of Kv1.3 for use as immunosuppressants; these agents could mimic the interactive surface of the toxins, in addition to taking advantage of the 4-fold symmetry of the channel.

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