Ion Channels

Semisynthesis of a Functional K^+ Channel**

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Potassium channels conduct K^+ ions selectively at very high rates.[1] They accomplish this task through a structural element in their pore called the selectivity filter. The selectivity filter replaces the water molecules that normally surround a hydrated potassium ion with protein atoms, which are predominantly carbonyl oxygen atoms from the main chain.[2,3] For this reason, techniques of chemical synthesis rather than conventional mutagenesis are required if we wish to alter the selectivity filter to understand the details of its function.

In a previous study, we produced a semisynthetic KcsA K^+ channel to enable chemical modifications of the selectivity filter.[4] This semisynthetic KcsA K^+ channel contains residues 1 to 125 of the 160-amino-acid protein. Similar to the equivalent recombinant (and truncated) KcsA K^+ channel, it forms a stable tetramer and binds agitoxin 2.[5,6] Thus, the semisynthetic and recombinant channels appear to be the same. These truncated channels, however, are not very useful for studies of ion conduction because, in contrast to the full-length KcsA channel, they do not give measurable single-channel currents in planar lipid membranes.[4] There is a simple reason for the failure: the semisynthetic channel did not include the 35 C-terminal amino acids that apparently are required to open the pore. The most likely explanation is that the probability of the open state becomes too low for single-channel recording methods when the C-terminus is absent.[4] This problem was not simple to solve, because addition of the missing 35 amino acids brought the length of the synthetic fragment beyond the practical limit for solid-phase peptide synthesis (SPPS).[7] In this study, we sought to remedy this limitation by identifying mutations within the pore that would

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favor channel opening even in the absence of the C-terminal residues.

Our rationale for identifying mutations is based on the idea that a gating hinge is important for the conformational change leading to pore opening in K^+ channels.[8,9] Closed (KcsA) and opened (MthK) K^+ channels[10] are shown in Figure 1a and b, respectively. The major difference in these structures lies in the conformation of the inner helices, which are straight in KcsA and bent in MthK. The fact that the position of the bend (colored red in Figure 1) is conserved as glycine in many K^+ channels (Figure 1c) implies that it may serve as a hinge point. The gating hinge and its vicinity thus seemed to be a promising target for mutations that might stabilize the open conformation of KcsA.

We introduced glycine or proline residues since they have a tendency to alter the stability of a straight α-helix. Mutant channels with single-site substitutions from position 98 to 104 were expressed in *Escherichia coli*, purified, and reconstituted into lipid vesicles and then planar lipid bilayers. Two mutants, S102P and A98G, exhibited prolonged mean open times compared to wild-type channels in single-channel records (Figure 2a). Under conditions in which the mean open time for the wild-type channel is 1.6 ms, the S102P and A98G mutants had mean open times of 162 and 8.2 ms, respectively (see the Supporting

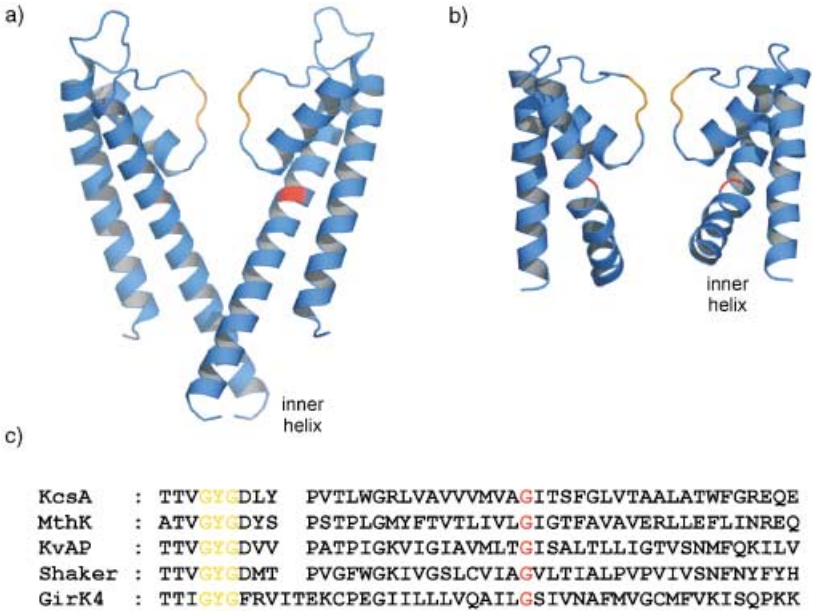

Figure 1. The gating hinge of potassium channels. a) Structure of the KcsA K^+ channel (pdb code: 1K4C). b) Structure of the MthK K^+ channel (pdb code: 1LNQ). For clarity, only two subunits of the KcsA and MthK tetramers are shown. c) Sequence alignment of the inner helices of the KcsA (gi1089906), MthK (gi21542150), KvAP (gi38605092), Shaker (gi13432103), and GIRK4 (gi976203) K^+ channels. The conserved GYG sequence in the selectivity filter is colored gold, while the gating hinge glycine is colored red.

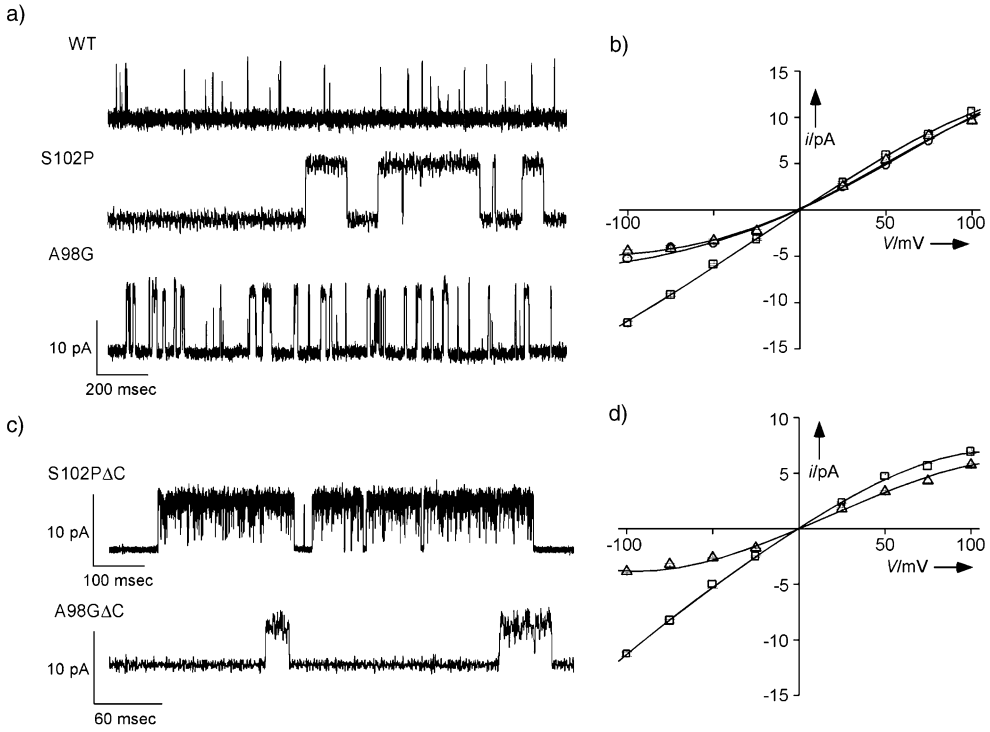

Figure 2. Representative single-channel traces (a) and *i–V* curves (b) for full-length wild-type KcsA (○), KcsA-S102P (□), and KcsA-A98G (△). Representative single-channel traces (c) and *i–V* curves (d) for the C-terminally truncated KcsA-S102PΔC (□) and KcsA-A98GΔC (△). Single-channel currents were recorded at +100 mV in symmetrical 150 mM K^+ solutions. Each data point in the *i–V* curves represents the average ± SE ($n \geq 3$) from at least two separate planar lipid membranes. The solid lines in the *i–V* plots have no theoretical meaning.

Information). The current–voltage (*i–V*) curves for wild-type and mutant channels in 150 mM KCl show that A98G is almost indistinguishable from wild type, but that conductance is higher at negative voltages for S102P (Figure 2b).

Since our aim is to identify a truncated KcsA K^+ channel that will open in planar lipid membranes, we used chymotrypsin to remove amino acids 126 to 160.[2] Following chymotrypsin cleavage, the truncated channels were purified by gel filtration chromatography, reconstituted, and analyzed in planar lipid membranes. Mutants A98GΔC (ΔC for truncation) and S102PΔC both exhibited single-channel opening (Figure 2c). The much shorter approximate mean open times following truncation of the C terminus (2.90 ms for A98GΔC mutant, Figure 4d) indicate that the C-terminus is still important for gating in these mutants. Nonetheless, the inner helix mutations appear to have shifted the gating equilibrium sufficiently toward the open conformation that single channels are now detectable in the absence of the C-terminal residues. The open-channel properties are also affected by truncation: the ΔC forms of both mutants showed two conductance states of about 60 and 45 pS (A98GΔC) and 70 and 50 pS (S102PΔC), measured as a cord conductance at +100 mV, and the *i–V* curves showed a reduced conductance at positive voltages compared to the full-length mutants (compare Figure 2b and d). The influence of the C-terminus on conductance may well reflect motions of the inner helices that are too rapid to resolve in the electrophysiological recording system.

Figure 3a outlines the procedure for semisynthesis of the KcsA K^+ channel.[4] Of the two mutations identified above, we chose to incorporate A98G into the semisynthetic channel because it migrates as a stable tetramer in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE, see the Supporting Information). The semisynthesis consists of two steps: an expressed protein ligation (EPL) reaction for the assembly of the polypeptide, and a folding reaction to form the KcsA tetramer. EPL enables two polypeptides, one recombinant and one synthetic, to be linked through a peptide bond.[11] The chemistry requires that one of the peptides contain an α-thioester and the other an N-terminal cysteine residue. Position 69 was chosen as the ligation site, which necessitates a cysteine rather than a serine residue (S69C mutant) in the final product. The S69C mutant was found to have similar stability to the wild-type protein, and both migrate as tetramers in SDS-PAGE analysis at room temperature (data not shown). In our previous study, threonine 74 was used as the ligation site,[4] but the resulting T74C mutant exhibited reduced stability, as judged by SDS-PAGE analysis.

The desired polypeptide was assembled from two fragments: a peptide α-thioester, referred to as the N-peptide, corresponding to residues 1 to 68 of KcsA, and a peptide containing an N-terminal cysteine residue, referred to as the C-peptide, corresponding to residues 69 to 122. The N-peptide was obtained by recombinant expression with a dual-fusion strategy, as described previously.[4] The C-peptide was obtained by using optimized Boc-based SPPS (Boc = *tert*-butoxycarbonyl) and purified as described previously.[4] The S69C and A98G mutations were incorporated into the C-peptide during synthesis. Ligation was carried out in the presence of 1% SDS, which was necessary to keep the N- and C-peptides soluble during the course of the reaction. The

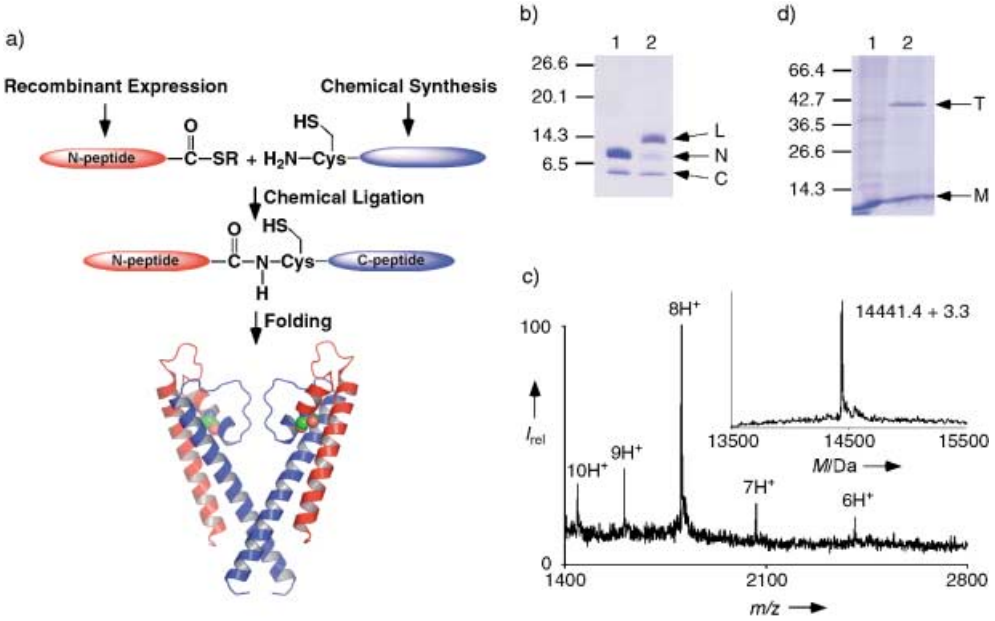

Figure 3. Semisynthesis of a functional K^+ channel. a) The KcsA polypeptide was assembled by expressed protein ligation of a recombinant N-peptide (residues 1–68, red) and a synthetic C-peptide (residues 69–122, blue). The product was then folded into the native tetramer by using lipid vesicles and purified by affinity and gel-flitration chromatographies. The ligation site, position 69, is depicted in space-filling form. b) SDS-PAGE analysis (15%) of the ligation reaction at 0 min (lane 1) and 24 h (lane 2) showing the C-peptide (C), N-peptide (N), and ligation product (L). c) ES-MS of the purified ligation product (inset: reconstructed spectrum; expected mass 14438.0 Da). d) SDS-PAGE analysis (12%) of the semisynthetic channel before (lane 1) and after (lane 2) addition of lipids. The monomer (M) and the tetramer (T) of semisynthetic KcsA are indicated.

ligation reaction was initiated by the addition of 2% thiophenol and incubation at 37°C. The reaction, progress of which was monitored by SDS-PAGE, was around 50% complete after 4 h and almost complete after 24 h (Figure 3b). Analysis of the ligation product by electrospray mass spectrometry (ES-MS) after purification by reverse-phase high-pressure liquid chromatography (RP-HPLC) yielded a signal that was consistent with the expected mass (Figure 3c).

Unfolded monomeric KcsA can be folded into a tetramer by using lipid vesicles. The ligation product was folded by a published lipid-based folding protocol with minor modifications.[4,12] Folding was confirmed by identification of the tetramer in SDS-PAGE (Figure 3d). The N-peptide used in the ligation reaction was engineered to carry an N-terminal His_6 tag, which facilitated two-step purification of the final product. In the first step, the folding mixture was passed over a column containing Co^{2+} ions to remove lipid vesicles from the protein, and in the second step gel-filtration chromatography was used to separate the tetramer from unfolded monomer and residual starting materials. In a typical small-scale synthesis, 0.06 mg of purified tetrameric semisynthetic KcsA channel was obtained from the ligation reaction of 0.7 mg of the N-peptide thioester and 1.0 mg of the C-peptide.

Purified semisynthetic KcsA K^+ channels containing the A98G mutation were reconstituted into lipid vesicles, and single-channel activity was tested in planar lipid membranes; single-channel recordings are shown in Figure 4a. As in the recombinant A98GΔC channel, two conductance states were observed, and the *i–V* curve (shown for the higher conductance state) is indistinguishable from that of the recombinant channel (Figure 4b). The histogram of open times has a mean value of 2.8 ms, which compares to a value of 2.9 ms for the recombinant channel (Figure 4c, d). These measurements indicate that the properties of the recombinant and semisynthetic KcsA K^+ channels are essentially the same.

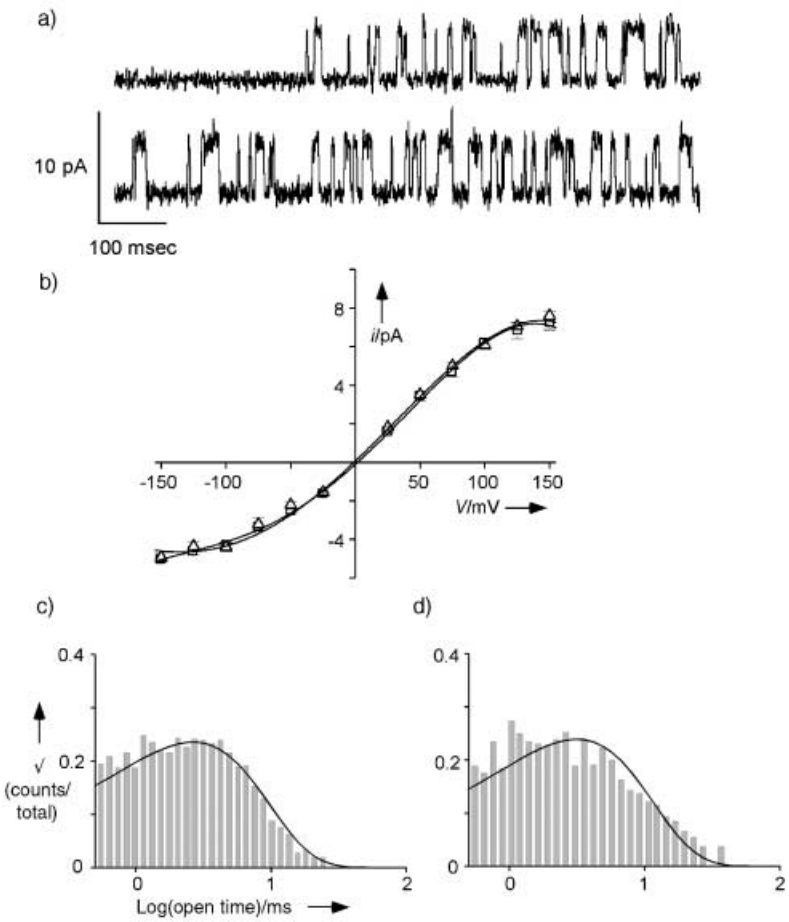

Figure 4. Single-channel activities of semisynthetic KcsA. a) Representative single-channel traces for the semisynthetic channel recorded at +100 mV in symmetrical 150 mM K^+ solution. b) *i–V* curve for the semisynthetic KcsA channel (△) and recombinant KcsA-S69C/A98GΔC channel (□). Single-channel currents were recorded in symmetrical 150 mM K^+ solutions, and each data point represents the average ± SE ($n \geq 3$) from at least two separate planar lipid membranes. Open-time histograms for semisynthetic KcsA (c) and recombinant KcsA S69CΔC channel (d) at +100 mV in symmetrical 150 mM K^+ solutions. Solid lines represent single exponential fits to the data (see the Supporting Information).

We have successfully produced a functional semisynthetic K^+ channel in which the region of greatest interest, the selectivity filter, has been produced by chemical synthesis. These procedures provide a way to study the effects of main-chain chemical modifications. Since K^+ ions in the filter interact almost exclusively with main-chain carbonyl oxygen atoms, these methods are necessary for the production of channels in which each of the four sites within the filter have been modified. The possibilities for modification are numerous and will be reported in due course.

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