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Semisynthesis and Folding of the Potassium Channel KcsA

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Abstract: In this contribution we describe the semisynthesis of the potassium channel, KcsA. A truncated form of KcsA, comprising the first 125 amino acids of the 160-amino acid protein, was synthesized using expressed protein ligation. This truncated form corresponds to the entire membrane-spanning region of the protein and is similar to the construct previously used in crystallographic studies on the KcsA protein. The ligation reaction was carried out using an N-terminal recombinant peptide α-thioester, corresponding to residues 1−73 of KcsA, and a synthetic C-terminal peptide corresponding to residues 74−125. Chemical synthesis of the C-peptide was accomplished by optimized Boc-SPPS techniques. A dual fusion strategy, involving glutathione-S-transferase (GST) and the GyrA intein, was developed for recombinant expression of the N-peptide α-thioester. The fusion protein, expressed in the insoluble form as inclusion bodies, was refolded and then cleaved successively to remove the GST tag and the intein, thereby releasing the N-peptide α-thioester. Following chemical ligation, the KcsA polypeptide was folded into the tetrameric state by incorporation into lipid vesicles. The correctness of the folded state was verified by the ability of the KcsA tetramer to bind to agitoxin-2. To our knowledge, this work represents the first reported semisynthesis of a polytopic membrane protein and highlights the potential application of native chemical ligation and expressed protein ligation for the (semi)synthesis of integral membrane proteins.

Introduction

Recent advances in the area of peptide ligation have extended greatly the size limits of proteins that can be chemically synthesized.[1] As a result, chemical synthesis has emerged as an important technique in protein structure/function studies. Native chemical ligation[2] and the related technique, expressed protein ligation (EPL),[3,4] have been extremely useful for the synthesis and semisynthesis, respectively, of proteins difficult or impossible to obtain by purely recombinant means.[1,5−7] While these techniques have been applied successfully to a large number of soluble proteins, their application to membrane proteins has been extremely limited.[8,9] There are several major technical challenges in the total synthesis or semisynthesis of membrane proteins by chemical ligation. The first problem involves the synthesis of large fragments of integral membrane proteins. From the standpoint of chemical synthesis, transmembrane segments of membrane proteins are generally rich in β-branched amino acids, which introduces significant difficulties during the chain assembly of the peptide.[10] Recombinant expression of such fragments is also problematic due to cell toxicity effects associated with the over-production of long hydrophobic polypeptides. The hydrophobic nature of these polypeptides is the source of another general problem in this area, namely solubilization and purification of such peptides can be nontrivial. Yet another challenge in the preparation of membrane proteins is the final protein folding step, which by necessity must be performed since the protein is being assembled from a series of presumably unstructured peptides. There have been only a few reports of refolding integral membrane proteins after complete unfolding.[11] In fact, bacteriorhodopsin is the only helical integral membrane protein that has been refolded after complete unfolding.[12] Perhaps as a result, the application of native chemical ligation to the synthesis of membrane proteins has been limited to relatively small (<100 aa) single-membrane spanning polypeptides mainly from viruses.[8,9,13] The total chemical synthesis or semisynthesis of a polytopic membrane protein has not yet been reported.

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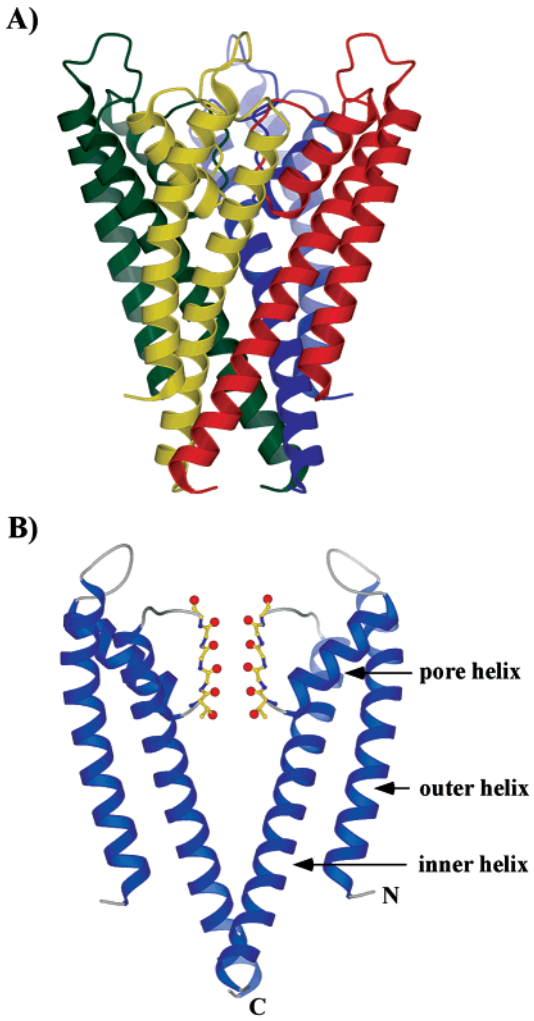

Figure 1. Structure of KcsA. (A) Shown is a side-on view of the tetrameric KcsA fragment (residues 1−125) used in these studies. (B) Two opposite subunits of the KcsA tetramer are shown. Residues 75−79 comprising the selectivity filter are shown in ball-and-stick representation.

Our interests are focused on potassium ion channels, integral membrane proteins that catalyze the selective conduction of potassium ions across biological membranes.[14] The high-resolution X-ray crystal structure of the bacterial potassium channel, KcsA, provided the first detailed look at a potassium channel.[15,16] KcsA, like all potassium channels, is a symmetric tetramer with the ion conduction pathway coincident with the central four-fold axis (Figure 1A). Each monomer consists of an N-terminal transmembrane helix followed by a short helix that leads to the selectivity filter followed by the C-terminal transmembrane helix. Sequence similarity between KcsA and other prokaryotic and eukaryotic potassium channels, particularly in the pore region, makes KcsA a good structural model for the pore region of all potassium channels (Figure 1B).[17]

Access to KcsA through chemical synthesis will enable the facile introduction of unnatural amino acids and peptide backbone modifications. This will be particularly useful in studies directed at understanding the molecular basis of potassium ion selectivity. Interactions between potassium ions and the channel responsible for ionic selectivity involve mainly the backbone carbonyl oxygen atoms of the selectivity filter (Figure 1B). As a result, a mutational analysis of these interactions is not possible by standard recombinant techniques.[18] In this report, we describe the use of expressed protein ligation for the semisynthesis of the KcsA potassium channel. A truncated version of the KcsA polypeptide was synthesized via the efficient native chemical ligation of two peptide fragments, one synthetic and one recombinant. Following the semisynthesis of the KcsA channel, we describe the folding of the channel to the tetrameric state and the characterization of the folded protein.

Results and Discussion

Synthetic Strategy. The KcsA construct employed in the crystallographic studies corresponded to the N-terminal 125 amino acids of the 160-amino acid protein.[15,16] The C-terminal truncation, which was required for crystallization, causes the channel to be predominantly in the closed conformation.[19] As a result, single-channel conductance measurements in lipid bilayers cannot be performed using this construct. Even with the lack of ionic conductance as a functional assay, the folded state of the construct can be accurately assessed by the ability of the channel protein to bind scorpion toxins.[17] Scorpion toxins bind over the pore at the interface between the four subunits and thus require an intact quaternary channel structure.[20,21] For synthetic reasons, this truncated version of KcsA was selected as the initial synthetic target. Importantly, this construct contains all the transmembrane portions of the channel and therefore tests the feasibility of chemically synthesizing and folding a polytopic membrane protein.

A semisynthetic strategy was developed that allowed KcsA-(1−125) to be generated from two component peptides by a single ligation reaction (Figure 2). Threonine-74 was selected as the ligation site as biochemical studies indicated that a Cys substitution at this position, which is required for the ligation chemistry, is structurally tolerated.[22] Furthermore, this ligation junction was desirable in a semisynthetic approach since all of the residues lining the central pore of the channel, including the selectivity filter, are located C-terminal to the ligation site. Thus, our semisynthetic strategy called for the generation of an N-terminal recombinant peptide α-thioester, corresponding to residues 1−73 of KcsA, and a synthetic C-terminal peptide corresponding to residues 74−125.

Chemical Synthesis of the KcsA 74−125. The C-terminal peptide is 52 amino acids long with 28% of the sequence consisting of β-branched amino acids (Ile, Thr, and Val). In anticipation that this would lead to problems in the chain assembly (i.e., kinetically slow couplings) a slightly modified version of in situ neutralization/2-(1H-benzo-triazol-1-yl)-1,1,3,3-

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(22) KcsA with the T74C mutation is tetrameric at room temperature as indicated by gel filtration chromatography and by $AgTx_2$ binding studies. The mutant has reduced sability under gel electrophoresis conditions and therefore SDS-PAGE analysis for this mutant was performed at 4 °C.

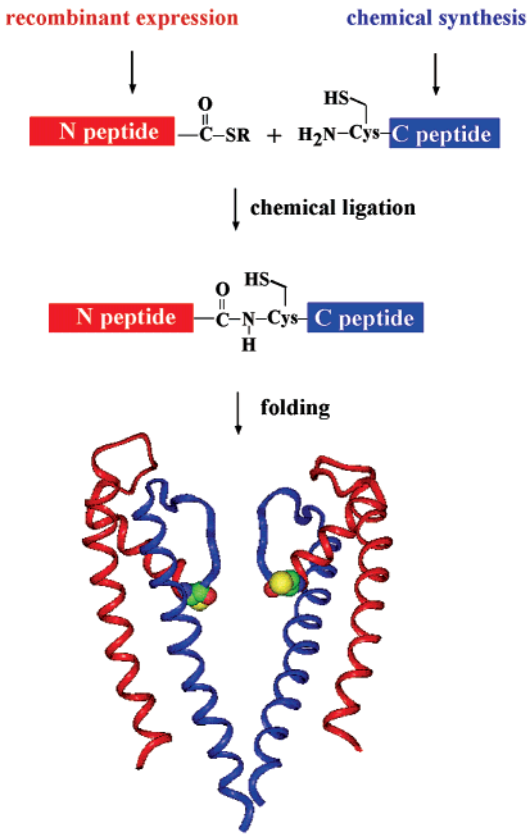

Figure 2. Semisynthesis of KcsA. The expressed protein ligation strategy used to generate KcsA. The KcsA monomer was synthesized by the ligation of a recombinant N-peptide (residues 1−73, red) and a synthetic C-peptide (residues, 74−125, blue). Two subunits of the KcsA tetramer are shown. The ligation site (position 74) is depicted in space fill.

tetramethyluronium hexafluorophosphate (HBTU) activation Boc-SPPS was employed.[23] All the β-branched amino acids were double-coupled using HBTU in DMF except for parts of the sequence containing consecutive β-branched amino acids, which were double-coupled using HBTU in DMSO.[24] Due to the extremely hydrophobic nature of the peptide, the crude material obtained after HF cleavage could not be dissolved in solvent systems that are generally used to solubilize long peptides, for example 1:1 MeCN/water, 0.1% TFA, or buffers containing 6 M guanidinium chloride. A number of solvent mixtures were screened (Table 1), and two of these, 3:1 TFE/DCM, 0.1% TFA, and 1:1 TFE/H_2O, 0.1% TFA, were identified as being capable of solubilizing the peptide at reasonable concentrations (1 mg/mL or higher). Of these two systems, only the latter was found to be compatible with efficient resolution of the crude by RP-HPLC (Figure 3). Using these synthesis, solubilization, and purification protocols, 9 mg of the C-peptide, at greater than 95% purity, could be obtained from 120 mg of the peptide crude.

Recombinant Expression of the KcsA 1−73 α-Thioester. A bacterial expression strategy was used to generate the N-peptide α-thioester fragment. α-Thioester groups can be introduced directly into recombinant proteins by thiolysis of the corresponding intein fusion proteins.[3,4] Accordingly, in initial

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Table 1: Solubility of C-peptide Crude in Various Solvents

solvent	solubility[a]
IPOH: H_2O (1: 1) + 0.1% TFA	+
MeOH + 0.1% TFA	+
MeOH: H_2O (1: 1) + 0.1% TFA	+ +
MeCN: MeOH (1: 1) + 0.1% TFA	+ + +
MeCN: IPOH (1: 1) + 0.1% TFA	+ + + +
TFE: H_2O (1: 1) + 0.1% TFA	+ + + + +
TFE: DCM (1: 3) + 0.1% TFA	+ + + + +
$CHCl_3$: MeOH: H_2O (4: 4: 1) + 0.1% TFA	+ +
$CHCl_3$: MeOH (2: 1) + 0.1% TFA	+ + + +
IPOH: H_2O (1: 1)	+
MeOH: H_2O (1: 1)	+
MeCN: H_2O (1: 1)	+ + + +
MeCN: MeOH (1: 1)	+
MeCN: IPOH (1: 1)	+
$CHCl_3$: MeOH (2: 1)	+
TFE: $CHCl_3$ (1: 3)	+ + + +

[a] For the determination of solubility, an aliquot of the crude was added to 1 mL of the solvent mixture, and the amount of the crude solubilized was determined by the 280 absorbance. Solubility was ranked from +, least soluble to + + + + + most soluble.

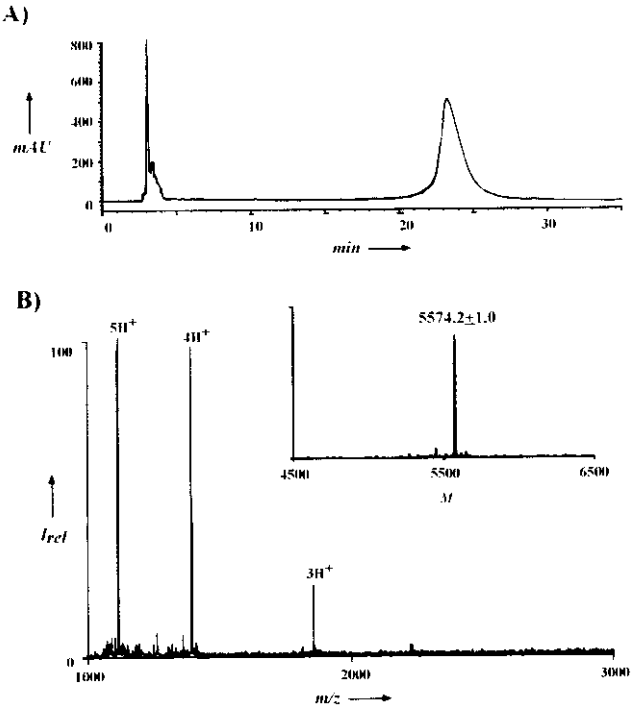

Figure 3. Purification of the C-peptide. (A) Analytical reverse-phase HPLC of the purified C-peptide (gradient 65−85% B over 30 min). (B) ES-MS of the purified C-peptide (inset, reconstructed spectrum, expected mass = 5572.5).

studies, we attempted to generate fusion proteins in which the N-peptide sequence was linked to either the *Mycobacterium xenopi* GyrA intein or the *Saccharomyces cerνisiae* VMA intein. In both cases, a variety of different expression conditions were examined; however, in no instance was the desired fusion protein detected (data not shown). We hypothesized that this was because the hydrophobic N-peptide sequence targeted the inteins to the secretory apparatus, which may have been toxic to the *Escherichia coli* cells. To circumvent this problem, a sandwich fusion approach was developed. Previous studies have shown that glutathione-S-transferase (GST) fusion often targets proteins to inclusion bodies,[25] which in principle should minimize or

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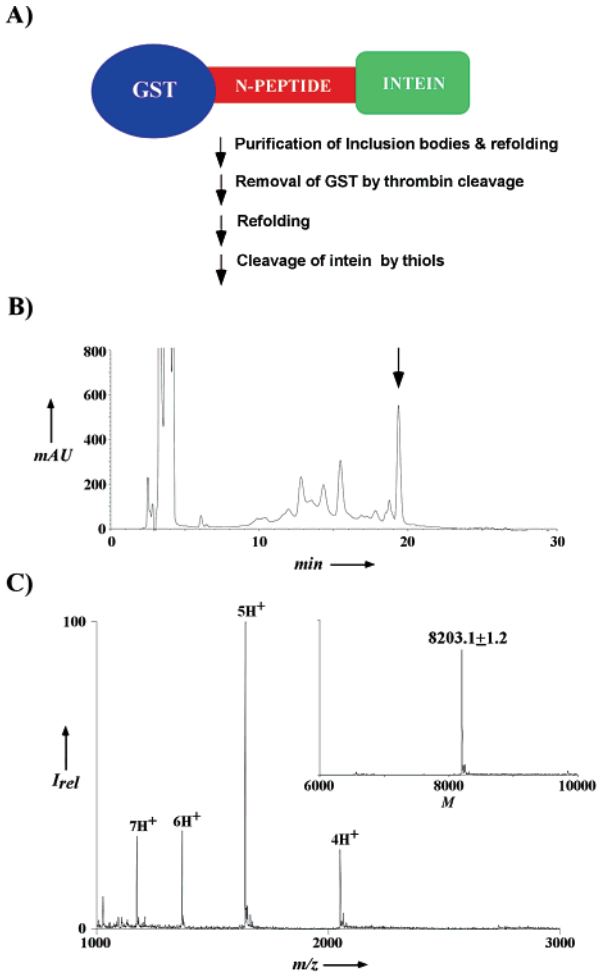

Figure 4. Purification of the N-peptide. (A) The dual-fusion strategy used for recombinant expression of the N-peptide α-thioester. (B) Analytical reverse-phase HPLC of the crude obtained from the cleavage reaction of the N-peptide−intein fusion with MESNA (gradient 50−90% B over 30 min). The N-peptide thioester is indicated. (C) ES-MS of the purified N-peptide (inset, reconstructed spectrum, expected mass = 8201.5).

eliminate any toxicity to the cells caused by protein expression. Accordingly, appending GST to the N-terminus of the KcsA−GyrA intein fusion was found to cause accumulation of the dual-fusion protein in inclusion bodies. Following cell lysis, these inclusion bodies were purified and solubilized using urea, and the fusion protein then refolded by dialysis (Figure 4A). At this point the GST tag was removed from the fusion protein by proteolysis with thrombin. The resulting KcsA−intein fusion was then cleaved using 2-mercaptoethanesulfonic acid (MESNA). Analysis of the crude cleavage mixture by RP-HPLC indicated that the thiolysis reaction had gone to >90% completion, indicating that refolding of the GyrA intein in the context of the dual fusion was quite efficient (Figure 4B). The resulting MESNA α-thioester peptide was purified using RP-HPLC and characterized by mass spectrometry (Figure 4C). Using this procedure, ∼2 mg of purified N-peptide α-thioester could be obtained per liter of bacterial culture. The dual fusion strategy and the refolding protocols described allow the recombinant production of long hydrophobic peptide α-thioesters and should have general applicability in the semisynthesis of integral membrane proteins.

Chemical Ligation. Given the very hydrophobic nature of the N- and C-peptides, conditions had to be identified under

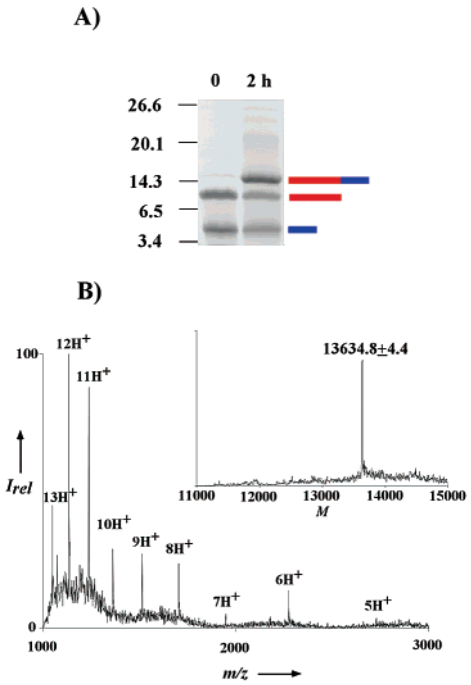

Figure 5. Chemical ligation of the N- and C-peptides. (A) SDS-PAGE gel showing the progress of the ligation reaction. The various peptides present in the reaction mixture are color-coded as in Figure 2. (B) ES-MS of the purified ligation product (inset, reconstructed spectrum, expected mass = 13633.0 Da).

which both molecules could be maintained in solution, thus allowing their efficient reaction via native chemical ligation. Trial ligation reactions were carried out in phosphate buffer at pH 7.6 to which either 50% TFE, 30% TFE + 4.8 M guanidinium chloride, 1% SDS, or 1% SDS + 8 M urea were added to help solubilize the reactant peptides. The ligation reactions were initiated by the addition of thiophenol to 2% and were analyzed by SDS-PAGE after overnight incubation at 37 °C. In the presence of 1% SDS, the peptides stayed soluble throughout the course of the reaction, whereas the peptides were found to slowly precipitate using the other reaction conditions tested. Subsequent studies revealed that prior lyophilization of the peptides in the presence of SDS allowed higher peptide concentrations to be achieved in the ligation mixture. Using these improved conditions, the ligation reaction was found to be both efficient and quite rapid, proceeding to around 80% completion after 2 h at 37 °C (Figure 5A). ES-MS analysis of the ligation product after RP-HPLC purification indicated that it had a mass consistent with the expected product, KcsA(1−125) (Figure 5B).

Folding of Semisynthetic KcsA. A key tenet of our synthetic approach was the ability to fold the tetrameric KcsA channel following ligation of the polypeptide fragments. Since there was no literature precedent for refolding an ion channel from a fully denatured state, conditions for refolding KcsA were first established using denatured recombinant protein. The folded state of KcsA can be conveniently assayed by SDS-PAGE since the native protein migrates as a tetramer on the gel. As reported previously, KcsA is remarkably stable, and the native tetramer persists under commonly used denaturing conditions such as 6 M guanidinium chloride or 8 M urea.[26,27] However, heating

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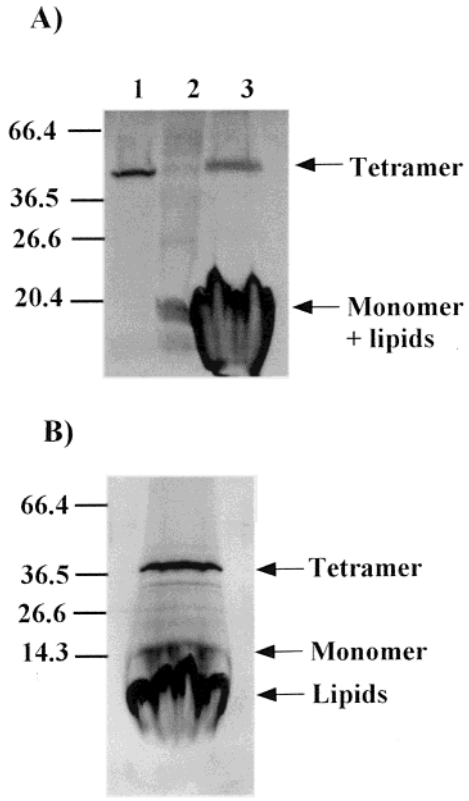

Figure 6. Folding of KcsA. (A) SDS-PAGE gel showing the refolding of recombinant KcsA. (Lane 1) native recombinant KcsA; (lane 2) unfolded KcsA; (lane 3) refolding of KcsA by lipids. (B) SDS-PAGE gel showing the folding of semisynthetic KcsA by lipids. The staining seen either overlapping the monomer band or slightly below the monomer band is due to the lipids used in the refolding reaction.

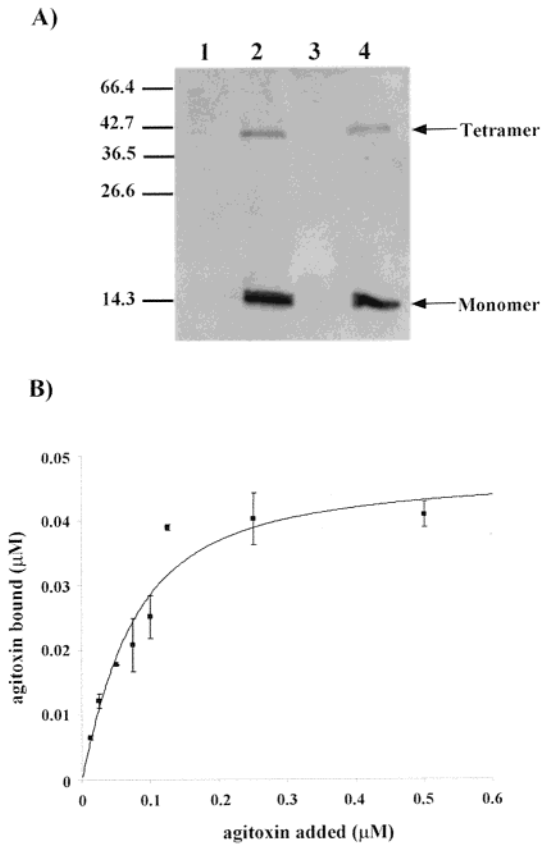

Figure 7. AgTx2 binding to semisynthetic KcsA. (A) The $AgTx_2$ affinity pull-down assay. SDS-PAGE gel showing: (lane 1) recombinant KcsA/control-resin; (lane 2) recombinant KcsA/$AgTx_2$-resin; (lane 3) semisynthetic KcsA/control-resin; (lane 4) semisynthetic KcsA/$AgTx_2$-resin. The KcsA protein is predominantly in the monomeric form as it was eluted from the $AgTx_2$ resin by boiling in SDS sample buffer. (B) Binding of $AgTx_2$ to semisynthetic KcsA. Quantity of $AgTx_2$ bound to semisynthetic KcsA immobilized on a cobalt resin as a function of the $AgTx_2$ concentration. Each point is the mean of two measurements.

native KcsA for 30 min at 100 °C in the presence of SDS fully unfolded the protein (Figure 6A). A large number of refolding conditions were examined using the heat-denatured protein. The only condition under which refolding to the tetrameric state occurred involved incorporation of the unfolded protein into lipid (soybean lipid) vesicles (Figure 6A). The yield of refolding was on the order of 30%.

For folding of the semisynthetic protein, the crude ligation mixture was diluted to a concentration of 0.1−0.2 mg/mL of the product and then further diluted into lipid vesicles. The dilution of the ligation mixture was necessary to reduce SDS concentration to 0.1% as refolding was not observed at higher SDS concentrations. SDS-PAGE analysis indicated that folding of the semisynthetic KcsA had taken place and to a level (around 30%) similar to that obtained using the denatured recombinant protein (Figure 6B). Direct folding using the crude ligation mixture was only possible when the extent of ligation was quite high. Otherwise, folding was possible only after HPLC purification of the ligation product. This is possibly due to inhibition of the folding process by the unligated peptides. Interestingly, a two-step protocol was necessary for folding HPLC purified protein. This involved initially dissolving the protein in 50% TFE:H_2O, 1.0% TFA and then dialysis into a solution containing 0.2% SDS. At this point the protein could be folded by reconstitution into lipid vesicles. The requirement of a two-step protocol is presumably due to differences in the unfolded state of the ligation product before and after HPLC purification.

Characterization and Purification of Semisynthetic KcsA. Agitoxin-2 ($AgTx_2$) binding was used to test the correctness of the folded structure of semisynthetic KcsA. $AgTx_2$ is a highly structured, 37-amino acid protein from scorpion venom.[28,29] It inhibits K^+ channels by binding to the pore entryway and inserting a Lys residue into the ion conduction path.[20,30,31] Even modest mutations near the selectivity filter abolish toxin binding, and therefore $AgTx_2$ binding provides a critical test of the structural integrity of the channel.[17,31] Furthermore, toxin-binding provides an assessment of the folded state of the entire channel preparation.[32] A mutant of $AgTx_2$ containing a $D^{20}\rightarrow C$ substitution was expressed and purified as described in the Experimental Methods. This material was then covalently immobilized onto iodoalkyl-agarose resin, using the free Cys residue. Control studies indicated that the folded recombinant protein bound to this $AgTx_2$-resin (Figure 7A, lane 2), while unfolded protein did not bind (data not shown). Similarly,

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semisynthetic KcsA was retained on the $AgTx_2$-resin only after lipid-mediated folding (Figure 7A, lane 4). Neither the recombinant nor the semisynthetic protein interacted with a control resin in which the iodoalkyl group was blocked with mercaptoethanol (Figure 7A, lanes 1, 3). We emphasize that the monomer on the gel (Figure 7A) is not the result of the monomer binding to the $AgTx_2$ resin but is a result of the dissociation of the tetramer caused by boiling in SDS that was employed to elute the channel from the resin. These pull-down experiments indicate that the refolded semisynthetic KcsA is capable of binding specifically to $AgTx_2$.

For quantitative studies of $AgTx_2$ binding, a variant of KcsA with an N-terminal His_6 tag was prepared by using semisynthesis protocols identical to those described above (see Experimental Methods for details). The His_6-tag enabled immobilization of the channel on a cobalt resin, and quantitative toxin binding studies were carried out using ^{3}H-labeled $AgTx_2$ (Figure 7B). Equilibrium binding measurements indicated that $AgTx_2$ bound semisynthetic KcsA with an equilibrium dissociation constant, K_d, of 47 $\pm$ 13 nM. A K_d of 620 nM has been estimated for $AgTx_2$ binding to full-length recombinant KcsA.[17] The difference seen in the K_d values could be either due to truncation of the C-terminus in the semisynthetic channel or due to presence of lipids during the toxin binding assays with semisynthetic KcsA. In either event, these $AgTx_2$ binding measurements clearly indicate that the semisynthetic channel has assumed the native KcsA fold.

The $AgTx_2$ affinity column used for the pull-down assay provided an easy means to purify the folded semisynthetic KcsA. The crude folding mixture was passed over the $AgTx_2$ column, and the unfolded protein washed off. The bound semisynthetic channel was then eluted from the $AgTx_2$ resin by multiple washes with high salt-containing buffer. In a typical small-scale synthesis, 0.1 mg of the purified, folded protein was obtained using this purification protocol, starting with a ligation reaction containing 0.5 mg of the C-peptide and 0.72 mg of the N-peptide. There is every reason to expect that milligram amounts of folded channel can be obtained upon scale-up.

Conclusions

In conclusion, we have succeeded in the semisynthesis of a truncated version of the KcsA potassium channel. The KcsA polypeptide was obtained by the native chemical ligation of a recombinant N-terminal peptide and a chemically synthesized C-terminal peptide. Following ligation, the nascent polypeptide was folded into the tetrameric state by incorporation into lipid vesicles, and the correctness of the folded state was verified by the ability of the KcsA tetramer to bind to $AgTx_2$. This study is the first example of the (semi)synthesis of a polytopic membrane protein. It is likely that the various synthetic and biosynthetic protocols developed herein will be applicable to the semisynthesis of other ion channel proteins.

Sequences forming the central pore of KcsA, including the selectivity filter, are all localized to the C-terminal peptide that was chemically synthesized. Hence, the semisynthesis protocols developed in this report will allow biophysical probes, noncoded side-chains, and backbone modifications to be site-specifically introduced into these critical regions of the channel. As noted above, single-channel analysis cannot be performed with the current KcsA construct due to the truncation of the C-terminus that causes the channel to be in the closed conformation. While the synthesis of full-length KcsA is actively being pursued, it is important to emphasize that the current truncated construct is perfectly suitable for bulk measurements that relate to the structure or liganded state of the protein. Indeed, the quantitative $AgTx_2$-binding studies serve to illustrate this point. A number of pharmacological agents are known to bind to the pore domain of human potassium channels,[15] the structures of which are expected to be similar to KcsA.[33] Thus, prudent incorporation of spectroscopic probes into the pore domain of KcsA (or mutants thereof) should provide valuable biosensors for the high-throughput screening for medically relevant compounds.

Materials and Methods

General Methods. All amino acid derivatives and resins were purchased from Novabiochem (San Diego, CA) and Peninsula Laboratories (Belmont, CA). All other chemicals were purchased from Sigma-Aldrich Chemical Co (St. Louis, MO). Restriction endonucleases were purchased from New England Biolabs (Beverly, MA) and thrombin was purchased from Boehringer Mannheim (Indianapolis, IN). Analytical RP-HPLC was performed on a Hewlett-Packard 1100 series instrument with 214 and 280 nm detection. Semipreparative and process RP-HPLC was routinely performed on a Walters DeltaPrep 4000 system fitted with a Waters 486 tunable absorbance detector. All RP-HPLC runs used linear gradient of buffer A (water, 0.1% TFA) versus buffer B (9:1 MeCN/water, 0.1% TFA). Mass spectrometric analysis was routinely applied to all synthetic peptides and components of reaction mixtures. Electrospray mass spectrometry (ES-MS) was performed on a Sciex API-100 single quadrupole electrospray mass spectrometer.

Chemical Synthesis of KcsA 74−125 (C-Peptide). The peptide, H_2N-CTVGYGDLYPVTLWGRLVAVVVMVAGITSFGLVTAALA-TWFVGREQERRGKG-COOH was synthesized by manual SPPS (0.35 mmol scale) on a PAM resin using a slightly modified version of the in situ neutralization/HBTU activation protocol for Boc chemistry.[23] All the β-branched amino acids in the sequence were double-coupled (2 $\times$ 10 min coupling time). The following β-branched amino acids T,[75] V,[76] V,[84] T,[85] and V[93]−V[95] were double-coupled using HBTU in DMSO.[24] Following chain assembly, global deprotection and cleavage from the resin was achieved by treatment with anhydrous HF containing 4% v/v *p*-cresol, 1 h at 0 °C. Following removal of HF, the crude peptide product was precipitated using cold anhydrous Et_2O, washed thoroughly with Et_2O containing 5% β-mercaptoethanol, and then dissolved in 50% TFE:H_2O containing 0.1% TFA and lyophilized. A fraction of the crude peptide (120 mg) was purified by semipreparative RP-HPLC (Vydac C4, 5 μm, 10 mm $\times$ 250 mm, flow rate 5 mL/min) using a linear gradient of 50−65% B over 60 min to give 9 mg of purified peptide. The purified peptide was characterized as the desired product by ES-MS [observed mass = 5574.2 $\pm$ 1.0; expected mass (average isotopic mass) = 5572.5].

Expression of the KcsA 1−73 α-Thioester (N-Peptide). A DNA fragment corresponding to the N-peptide, residues Met1 → Ala73 of KcsA with the following amino acid substitutions, Pro2→Ala, Gln58→Ala, Thr61→Ser, and Arg64→Asp, was amplified by PCR and cloned into the pTXB3 expression vector (New England Biolabs, Beverly, MA) using the *Nco*I and *Sap*I restriction endonuclease sites. (The mutations were required to allow agitoxin binding.[17]) This resulted in fusion of the N-peptide with the *Mycobacterium xenopi* GyrA intein. The N-peptide-intein construct was then PCR amplified and fused to GST. The 5′ primer was designed to code for either a thrombin site followed by a Gly_6 linker or a thrombin site followed by a His_6 linker and a factor Xa site before Met1 of the N-peptide. The 3′ primer contained an *Eco*RI site that was introduced after the stop codon of the GyrA intein-CBD (chitin binding domain) gene. The PCR-amplified

(33) Lu, Z.; Klem, A. M.; Ramu, Y. *Nature* **2001**, *413*, 809−813.

DNA was cloned into the pGEX4T-2 expression vector (Amersham Biosciences, Piscataway, NJ) using the *Bam*HI and *Eco*RI restriction endonuclease sites. This cloning strategy resulted in the generation of two expression plasmids each coding for a sandwich fusion of the type GST-$(XX)_6$-[N-peptide]−[intein-CBD] where XX_6 refers to either a poly-Gly or poly-His linker. The plasmids were then transformed into BL21(DE3) *E.coli* cells, and the transformants were grown in LB medium to an O.D of 1−1.5 and protein expression was induced by the addition of IPTG to 1 mM for 3 h. Cells were harvested and washed with 50 mM Tris, pH 7.5, 0.2 M NaCl before being frozen at −80 °C.

Purification of the N-Peptide. The following protocol was used for both fusion proteins, that is, with the poly-Gly or poly-His linker. Cells from 1 L of culture were thawed and resuspended in 100 mL of buffer containing 10 mM Tris, pH 7.5, 5 mM EDTA, and 1 mM PMSF. Lysozyme was added to a concentration of 0.05 mg/mL, and the cells were incubated at 4 °C with gentle stirring. After 30 min, $MgCl_2$ was added to 10 mM, DNAse was added to 5 μg/m,L and the cells were lysed by sonication, 5 × 30 s bursts using a Branson model 450 sonifier at maximum power. After sonication, Triton X-100 was added to 1% (v/v) and the cell lysate incubated at room temperature for 30 min. The soluble fraction was separated by centrifugation at 12000*g* for 15 min at room temperature. The insoluble fraction, containing the inclusion bodies, was washed once with 100 mL of buffer containing 50 mM Tris, pH 7.5, 1% Triton X-100 (v/v), 1 mM EDTA, 2 M urea. The insoluble fraction obtained after the 2 M urea wash was greatly enriched in the fusion protein and was solubilized in 100 mL of buffer containing 6 M urea (50 mM Tris, pH 7.5, 1% Triton X-100 (v/v), 1 mM EDTA, 6 M urea). Any material still insoluble at this stage was removed by centrifugation at 12000*g* for 15 min at room temperature. The solubilized fusion protein was then refolded by dialysis against 2 L of 50 mM Tris, pH 7.5, 0.2 M NaCl, 1% Triton X-100 (v/v), 1 mM EDTA for 24 h at 4 °C. After dialysis, any protein precipitate present was removed by centrifugation at 12000*g* for 15 min at room temperature. The GST tag was then removed by thrombin digestion (40 units) in the presence of 5 mM $CaCl_2$ for 12 h at room temperature. Thrombin cleavage invariably resulted in precipitation of the cleavage products. The protein precipitate was therefore redissolved by adding urea (solid) to a final concentration of 8 M. The concentration of urea in the protein solution was reduced by dialysis against 1 L of 50 mM Tris, pH 7.5, 0.2 M NaCl, 1% Triton X-100 (v/v), 1 mM EDTA for 4 h at room temperature. Following dialysis, any protein precipitate present was removed by centrifugation and the N-peptide−intein fusion was bound to chitin beads (20 mL) equilibrated with 50 mM Tris, pH 7.5, 0.2 M NaCl, 1% Triton X-100 (v/v), 1 mM EDTA. After incubation at room temperature for 30 min with gentle stirring, the chitin beads were extensively washed with buffer containing 50 mM Tris, pH 7.5, 0.2 M NaCl. The chitin beads were then re-suspended as a 50% slurry in 50 mM Tris, pH 7.5, 0.2 M NaCl and 2-mercaptoethanesulfonic acid (MESNA) was added to 0.1 M. The slurry was incubated at room temperature for 16 h. Due to the hydrophobic nature of the N-peptide, all the peptide that was cleaved from the intein was nonspecifically adsorbed on the chitin beads. Following cleavage with MESNA, the chitin beads were extensively washed with H_2O to remove all salts and any remaining Triton X-100 present. The adsorbed proteins were then stripped off the chitin beads by gently stirring with a solution containing 50% TFE: H_2O, 0.1% TFA for 30 min at room temperature. The resulting solution was filtered initially through glass wool to remove the chitin beads and then through a 0.45 μM filter (Gelman, Ann Arbor, MI). The N-peptide α-thioester was then purified by preparative RP-HPLC (Vydac C4, 15 μm, 50 mm × 250 mm, flow rate 30 mL/min) using a linear gradient of 50−90%B over 45 min on a C4 column. This gave 2.0 mg of purified peptide, which was characterized as the desired product by ES-MS [observed mass = 8203.1 ± 1.2; expected mass (average isotopic mass) = 8201.5 for the N-peptide α-thioester with the Gly_6 linker and observed mass = 9139.8 ± 3.2; expected mass (average isotopic mass) = 9137.5 for the N-peptide α-thioester with the His_6 linker].

Native chemical Ligation. Prior to setting up a ligation reaction, 0.72 mg of the N-peptide α-thioester and 0.5 mg of the C-peptide were lyophilized with 1.5 and 0.5 mg of SDS, respectively. Co-lyophilizing the peptide with SDS enabled easy solubilization of the peptides in the ligation buffer. The two peptides were dissolved in 0.2 mL of ligation buffer containing 100 mM sodium phosphate, pH 7.6, and thiophenol was added to 2% (v/v). The reaction was performed at 37 °C with gentle stirring, and SDS-PAGE was used to monitor the progress of the ligation reactions. The ligation reactions were terminated by addition of β-mercaptoethanol to 5% (v/v). For RP-HPLC purification of the ligation product, the reaction mixture was diluted 1→10 using 50% TFE: H_2O containing 0.1% TFA and then separated by using analytical RP-HPLC (Vydac C4, 5 μm, 4.6 mm × 150 mm, flow rate 1 mL/min) and a linear gradient of 50−100% B over 30 min. The ligation products were identified as the expected KcsA (1−125) analogues by ES-MS [observed mass = 13634.8 ± 4.4; expected mass (average isotopic mass) = 13633.0 for semisynthetic KcsA with the Gly_6 linker at the N-terminus and observed mass = 14648.32 ± 3.8; expected mass (average isotopic mass) = 14642.2 for semisynthetic KcsA with the His_6 linker at the N-terminus].

Folding of Semisynthetic KcsA. The folding reaction was generally carried out on the ligation mixture without purification. The ligation reaction was diluted 1→5 using lipid buffer (100 mM Tris, pH 7.5, 200 mM NaCl, 15 mM KCl, 10% glycerol, 10 mM DTT) and then added to an equal volume of lecithin (type IV-S, Sigma) vesicles (30 mg/mL in lipid buffer). The SDS/lipid ratio (0.1% SDS to 15 mg/mL of lipid) seems to be critical as refolding was not observed at higher SDS/lipid ratios. The mixture was then sonicated in a water bath sonicator for 5 min and then incubated at room temperature. The extent of folding was monitored by the appearance of a tetramer band on SDS-PAGE.

Generation of the $AgTx_2$ Resin. $AgTx_2$ containing the D^{20}→C substitution was expressed in *E. coli* and purified in the dimeric form as described previously.[34] The dimeric toxin was converted into the fully reduced monomer by treatment with 10 mM DTT for 2 h at 37 °C. The unfolded toxin was purified by semipreparative RP-HPLC (Vydac C18, 10 μm, 10 mm × 250 mm, flow rate 5 mL/min) using a linear gradient of 15−40% B over 60 min. The unfolded toxin was dissolved in 50 mM sodium phosphate, pH 8.0, 5 mM EDTA and incubated at room temperature for 30 min, to enable refolding of the toxin through the formation of the three native disulfide bonds. The toxin was then coupled to sulfolink resin (Pierce) using the free sulfhydryl group of Cys20. This reaction was carried out at room temperature with gentle stirring and monitored by RP-HPLC analysis of the supernatant. After 5−6 h of reaction most of the toxin had been coupled onto the resin. Any unreacted iodoacetamide groups on the resin were then capped by treatment of the resin with 1% β-mercaptoethanol for 1 h at room temperature. Following the capping reaction, the resin was extensively washed with 50 mM sodium phosphate, pH 8.0, 5 mM EDTA and stored as a 50% slurry at 4 °C. A control resin was also produced in which the iodoacetamide groups were simply capped with 1% β-mercaptoethanol.

The "Channel Pull-Down" Assay. For the channel pull-down assay, the solution containing the folded semisynthetic channel or the recombinant channel was diluted 1→5 with assay buffer (50 mM Tris, pH 7.5, 50 mM KCl, 10 mM decyl maltoside (DM)). $AgTx_2$ resin (25−50 μL) was then added and the slurry incubated at room temperature for 30 min with gentle shaking. The resin was then washed extensively with the assay buffer. Due to the tight binding of the channel to the toxin, the channel protein bound was eluted by boiling the resin in 1× SDS sample buffer. For purification of the tetrameric channel, the bound channel was eluted from the toxin by washing with 3×

(34) Aggarwal, S. K.; MacKinnon, R. *Neuron* **1996**, *16*, 1169−1177.

volumes of elution buffer (50 mM Tris, pH 7.5, 1.0 M KCl, 10 mM DTT, 10 mM DM).

Binding Affinity Measurement of $AgTx_2$. Equilibrium binding measurements of $AgTx_2$ to the semisynthetic KcsA were performed using the ^{3}H-labeled $AgTx_2$ and the KcsA variant carrying the N-terminal His_6 tag. Tritiated *N*-ethylmaleimide (NEN Life Sciences, Boston, MA) was conjugated to $AgTx_2$ containing the $D^{20}\rightarrow C$ mutation as described previously.[34] For the K_d measurements, semisynthetic KcsA was immobilized on cobalt resin (Clontech, Palo Alto, CA) through the N-terminal His tag. Binding assays were carried out in a 120-μL volume of assay buffer (50 mM Tris, pH 7.5, 100 mM KCl, 2.5 mg/mL BSA, 10 mM DM) and 1μL of the cobalt resin containing approximately 0.25 μg of protein.[17] Tritiated $AgTx_2$ was added at different concentrations and the mixture incubated at room temperature for 30 min. with gentle agitation after which the unbound toxin was separated from the resin by filtering through a GF/C glass microfiber filter (Whatman, Fairfield, NJ). The resin on the filter was rinsed with assay buffer, and the radioactivity retained on the filter was determined using a scintillation counter. The amount of nonspecific binding at each toxin concentration was determined from a control sample containing an excess (0.1 mM) of unlabeled $AgTx_2$. The equilibrium dissociation constant, K_d, was determined from the equation:

$$[\text{bound}] = \frac{([\text{total}] + A + K_d) - \sqrt{([\text{total}] + A + K_d)^2 - 4A[\text{Total}]}}{2}$$

where [bound] and [total] correspond to the concentration of bound and total $AgTx_2$ and A corresponds to the concentration of total channel present.

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