Wrestling with Native Chemical Ligation

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The ability to make polypeptides incorporating amino acids other than the 20 normally used for ribosomal protein synthesis offers numerous opportunities in basic and applied biology. Several approaches to the introduction of non-canonical amino acids have been developed, but they have been especially difficult to apply to membrane proteins. In this issue, Komarov and colleagues (1) report the semisynthesis of a full-length subunit of the potassium (K) channel KcsA, which can be assembled into an active homotetramer. Their improved approach facilitates the placement of unusual amino acids (or other building blocks) at any point in the polypeptide chain.

Relatively little work has been done on engineered membrane proteins, by comparison with, say, enzymes and antibodies. This deficiency stems from attributes inherent to proteins that reside in lipid bilayers, notably the poor solubility of the folded and unfolded polypeptides (and their fragments) in the absence of detergents or lipids. Ion channels and pores are particularly vital targets in the membrane protein field, contributing knowledge at the frontiers of our understanding of protein mechanisms and providing components for emerging applications in biotechnology. To progress beyond conventional mutagenesis, non-canonical amino acids have been introduced into ion channels by using amber codon suppression (2), and protein pores have been modified at predetermined sites, either covalently (3) or noncovalently (4). Both of these approaches have their boundaries. While the array of amino acids that is handled by ribosomes is impressive, there are limitations (5), and chemical modification must avoid nonspecific reactions and is difficult to apply at multiple sites (6). Therefore, the introduction of synthetic peptide or non-peptide segments into polypeptides by native chemical ligation (7) is a powerful complementary approach (8).

Ion channel subunits have been prepared directly by a single run of solid-phase peptide synthesis (SPPS), but in this case they have been rather small, for example, the 32-amino-acid transmembrane segment of the HIV-1 protein Vpu (9). The TASP (template-assisted synthetic protein) approach has allowed the preparation of larger molecules, for example, Vpu tetramers and pentamers (10), but there is little evidence that these fold into well-defined structures. Therefore, progress on the construction of membrane proteins by synthetic or semisynthetic approaches that involve native chemical ligation has been tracked with interest. For example, Clayton and colleagues achieved a heroic preparation of a mechanosensitive channel, MscL, by using chemical ligation to connect, sequentially, three fragments of 34, 57, and 55 residues prepared by SPPS (11). Yet larger subunits of membrane proteins should be accessible by ligating recombinant and synthetic fragments, *i.e.*, semisynthesis. Pioneering work in this area has been carried out by Francis Valiyaveetil, who has made several versions of KcsA, first at Rockefeller University with Tom Muir and Rod McKinnon, and now in his own laboratory at Oregon Health & Sciences University.

KcsA has become an important model system, because it was the subject of the first high resolution X-ray structure of any ion channel. In earlier studies (12), Valiyaveetil and colleagues built KcsA from

ABSTRACT An improved method for the semisynthesis of a potassium channel involving native chemical ligation allows the introduction of short sequences containing non-canonical amino acids at any position within the polypeptide chain. The work enhances the technology available for a range of fundamental investigations of membrane proteins and for applications of membrane channels and pores in biotechnology.
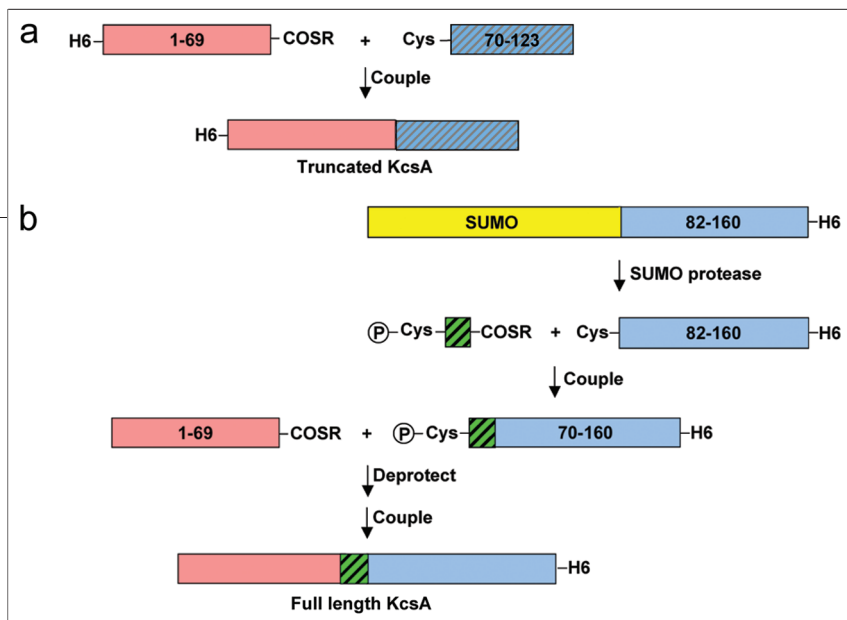
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Figure 1. Semisynthesis of KcsA. a) Example of a truncated subunit made by the earlier route (*12*). b) Full-length KcsA made by the new three-fragment approach (*1*). N-Terminal fragment, pink; C-terminal fragment, blue; central fragment, green; Sumo domain, yellow; fragment made by SPPS, crosshatched. The synthetic central peptide fragment (crosshatched, green) can be made to contain non-canonical amino acids or non-amino acid building blocks.

two fragments: an N-terminal recombinant piece (with a C-terminal thioester) and a C-terminal synthetic fragment (with an N-terminal cysteine) (Figure 1, panel a). The site of the junction was dictated by the desire to place non-canonical amino acids in the central "pore domain". Because fragments of >50 or so amino acids are tough to make by SPSS, the C-terminal fragment was limited in length and the semisynthetic KcsA was therefore truncated at its C termi-

nus. Consequently, the first proteins made by this approach were inactive, and conducting forms were obtained only after further mutagenesis.

A new semisynthesis strategy is presented here (*1*) that allows the production of full-length KcsA, but it requires the assembly of three fragments. The N- and C-terminal pieces are recombinant polypeptides, and the central region is a synthetic peptide of modest length (Figure 1, panel b).

In this way, SPPS is used only for a specific region of interest, which can be equipped with a molecular probe or functionally altered. Further, this region can be placed centrally. The key to success was the preparation of the C-terminal piece as a Sumo-fusion protein of the form SumoGlyGly$^\vee$-CysKcsA(82−160)-thrombin site-His tag. As predicted by the authors, on the basis of their knowledge of Sumo expression, the fusion protein was produced in abundance by *E. coli*. After solubilization with 1% (w/v) *N*-lauryl-sarcosine and the addition of Triton-X100 to 2% (v/v), the protein was purified by using a Co^{2+}-affinity column and then cleaved with Sumo protease after the Gly-Gly sequence to yield a His-tagged protein with an N-terminal cysteine residue, which was purified by reverse-phase HPLC. Thrombin and TEV protease failed to release N-terminal cysteine polypeptides from similar constructs, most likely because the expressed protein formed aggregates in the absence of a Sumo domain.

The C-terminal peptide was then coupled with a 10-fold excess of the synthetic central peptide thioester (70−81) in 1% Fos-12 (Figure 1, panel b). Thereafter, the synthesis resembles the earlier route (Figure 1, panel a) and uses many of the technical advances gained in that work, including producing the N-terminal recombinant polypeptide by a "sandwich" approach, carrying out the final ligation in lipid bilayers, refolding by diluting the full-length product from SDS into excess lipid vesicles, and purifying the resulting tetramer first on a Co^{2+}-column and then by gel filtration, both in *n*-dodecyl-β-ᴅ-maltoside. A different version of KcsA with an altered pore helix in which the central peptide encompassed residues 54−69 was made in a similar way.

The power of the approach can be appreciated when it is recognized that many of the fundamental functional properties of membrane proteins, including KcsA, are mediated by short sequences of 10−20 residues. In K channels such features include
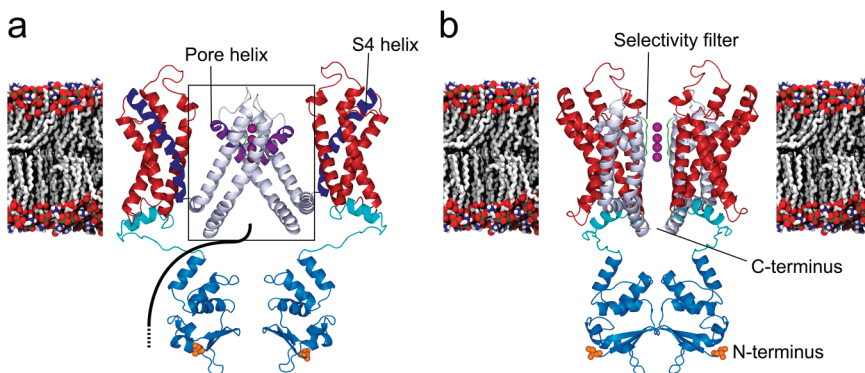

Figure 2. Functional sites in a voltage-gated K channel. Two views are shown of two of the four subunits in a chimeric K channel (PDB 2R9R). Short linear sequences that are largely responsible for specific functional properties of the channel are indicated: S4 helix, dark blue (voltage sensing); pore helix, purple (C-type inactivation); selectivity filter, green (selectivity for K$^+$ ions); N terminus, black (N-type inactivation [panel a], the inactivation peptide can be at the N terminus of a K channel subunit or on an accessory β subunit). These functional properties might be explored further by using subunits constructed by semisynthesis in which the designated sequences have been replaced by synthetic peptides or alternative structures. KcsA, featured in the present work, is a small K channel and comprises only the central region of the structure shown here (boxed on left).

ion selectivity, voltage (and other forms) of gating, inactivation, interactions with accessory proteins, subunit–subunit interactions, the binding of blockers including toxins, *etc*. (Figure 2). The three-part semisynthesis strategy might be used in studies of these features in the wider world of K channels for which there are ~70 human genes (*13*). The findings and indeed the technology will be applicable to many Na and Ca channels and other relatives of K channels. In the near future, we can expect to see additional advances in the semisynthesis of membrane proteins based on native chemical ligation, such as the use of Arg-tags to improve peptide solubility (*14, 15*). Further, channels and pores altered in this way will find applications in emerging technologies including sensing, DNA sequencing, drug delivery, and cell preservation (*16, 17*).

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