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In vitro synthesis, tetramerization and single channel characterization of virus-encoded potassium channel Kcv

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Abstract **Chlorella virus-encoded membrane protein Kcv represents a new class of potassium channel. This 94-amino acids miniature K^+ channel consists of two *trans*-membrane α-helix domains intermediated by a pore domain that contains a highly conserved K^+ selectivity filter. Therefore, as an archetypal K^+ channel, the study of Kcv may yield valuable insights into the structure–function relationships underlying this important class of ion channel. Here, we report a series of new properties of Kcv. We first verified Kcv can be synthesized in vitro. By co-synthesis and assembly of wild-type and the tagged version of Kcv, we were able to demonstrate a tetrameric stoichiometry, a molecular structure adopted by all known K^+ channels. Most notably, the tetrameric Kcv complex retains its functional integrity in SDS (strong detergent)-containing solutions, a useful feature that allows for direct purification of protein from polyacrylamide gel. Once purified, the tetramer can form single potassium-selective ion channels in a lipid bilayer with functions consistent to the heterologously expressed Kcv. These finding suggest that the synthetic Kcv can serve as a model of virus-encoded K^+ channels; and its newly identified properties can be applied to the future study on structure-determined mechanisms such as K^+ channel functional stoichiometry.**

Keywords: Cell-free protein synthesis; Ion selectivity; Kcv; Lipid bilayer; Potassium channel; Single channel

1. Introduction

Kcv is a *Paramecium bursaria* chlorella virus (PBCV-1)-encoded membrane protein that features the essential structure of the K^+ channels from prokaryotes and eukaryotes [1–3]. When expressed in *Xenopus* oocytes [1,3], mammalian HEK293 [4] cells and Chinese hamster ovary (CHO) cells [2], Kcv yields robust K^+ selective currents sensitive to block by Ba^{2+} and amantadine [1]. This virus-encoded K^+ channel is supposed to be present in the internal membrane of the viral particle. Upon fusion of the viral membrane with the host plasma membrane, it may depolarize the local plasma membrane, promoting the viral genome release into the host [5,6]. The Kcv activities may also be regulated by phosphorylation or dephosphorylation [7]. In addition, Kcv may also participate in other important functionalities. For example, it may thermodynamically couple with virus-encoded aqua-glyceroporin to simultaneously alter water conductance and driving force for water movement [8].

While the physiological roles of Kcv are being intensively investigated [4–6,8], its unique structure is becoming a new focus. Constituted by only 94-amino acids, this miniature Kcv is the shortest K^+ channel protein ever found. It possesses the most primitive structure of all K^+ channels: two *trans*-membrane α-helix domains intermediated by a pore domain with 61% similarity and 38% identity relative to the pore regions of many other K^+ channel proteins [1]. Most notably, the pore domain of Kcv is highly conserved at the K^+ selectivity filter sequence, TXXTXGFG, which is common to virtually all K^+ channels [1]. Recently, the site-directed mutagenesis in combination with electrophysiology study based on macroscopic current recordings of heterologously expressed protein has produced a wealth of information on channel structure-related properties of Kcv. For example, Kcv is a moderate voltage-dependent, inward rectifying channel with distinct kinetics features [9]; the short N-terminus of Kcv is a possible region that modulates channel conductance [4]; and the proline kink of TM1 at the membrane/aqueous interface may control the orientation of TM1 in the lipid bilayer, therefore play an important role in the time- and voltage-dependent inward rectification [10]. Moreover, novel approach using natural diversity analysis of the Kcv family encoded by chlorella viruses [3,11] has been used to identify key amino acids (at positions of 19, 54 and 66) affecting K^+ channel properties through long distance interaction [12]. In summary, these unique structural features suggest Kcv is a model channel in research on structure-determined molecular mechanisms.

In this paper, we report a series of studies which reveal newly identified properties relating to Kcv: cell-free synthesis, molecular stoichiometry, stability in strong detergent SDS and single channel characterization. Cell-free protein synthesis is a powerful protein production tool that has advantages over conventional techniques in time-efficiency, capability of synthesis with labeled amino acids (such as S35-methionine) and simplified protein purification; thus, it has been utilized to produce a broad series of membrane proteins including ion channels [13–16], neural receptors [17], ion pumps [18], drug transporters [19] and many bacterial protein pores [20–23]. In this report, we describe Kcv synthesis using coupled IVTT technique. Through co-synthesis and assembly of the wild-type Kcv and tagged variant, we were able to separate a series of homo- and hetero-oligomer by electrophoresis, and demonstrate a tetrameric Kcv stoichiometry, a common structure adopted by all known K^+ channels. Most notably, the

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tetrameric Kcv retains its functional integrity in SDS-containing solution. With this capability, the tetramer can be directly purified from polyacrylamide gel and form single K^+ channels in the lipid bilayer.

We are motivated to study Kcv due to its potential in elucidating the K^+ channel functional stoichiometry, a structural–functional relationship that regulates how the functions of the K^+ channel such as high K^+ selectivity depend on the individual contribution by each of the four identical subunits. It is known that the four identical subunits in a K^+ channel are symmetrically associated and work concertedly in conferring unique functions on channels [24–29]. Our interest is the modulation of channel properties by each subunit individually in a tetramer [30–32]. The strategy of this investigation is to detect hetero-channels that contain different numbers of functionally-inactive subunits. According to the findings in this report, the miniature K^+ channel Kcv is suitable to this investigation, since the hetero-Kcv proteins can separate in the gel and the Kcv tetramer purified from gel can function as a K^+ channel. These properties could be very useful for detecting each hetero-Kcv channel individually. Ultimately, we are able to investigate at the single channel level the impact of such hetero-Kcv structures on the channel properties.

2. Materials and methods

2.1. Kcv gene assembly and amplification

With the aid of software DNAWorks [33], the Kcv gene was optimized for *Escherichia coli* expression from its known amino acid sequence [3], and was assembled and amplified from oligonucleotides by PCR. All the oligonucleotides were limited to 50 nucleotides in length; the annealing temperature T_m, (evaluated from the GC content) of optimized oligonucleotides was 58 °C with a range of 1.6 °C; and the total codon usage score was 0.725. Unique *Nde*I and *Hind*III cloning sites (underlined below) as well as stop codons (lower case below) were also introduced. The forward and reverse strands were each assembled from seven overlapping oligonucleotides:

Forward strand
5′CATATGTTAGTGTTTTCTAAATTCCTGACC,
CGCACGGAACCATTTATGATCCACTTATTCATCTTAGCGA-TGTTTGT,
GATGATTTACAAGTTCTTTCCGGGTGGCTTTGAGAACAAC-TTCTCT,
GTGGCGAATCCGGCACAAAAAAGCGTCTTGGATTGATTG-TATTTATTT,
TGGTGTGACCACCCACTCTACCGTTGGTTTCGGTGATAT,
CTTACCAAAAACGACGGGCGCGAAACTGTGCACGATC
GCCCACATTGTGACGGTGTTCTTTATTGTTTTAACCCTG-tgataaG,
and reverse strand
5′AAGCttatcaCAGGGTTAAAACAATAAAGAAC,
ACCGTCACAATGTGGGCGATCGTGCACAGTTTCGC,
GCCCGTCGTTTTTGGTAAGATATCACCGAAACCAACGG-TAG,
AGTGGGTGGTCACACCAAAATAAATACAATCAATCCAAG-ACGCTTTT,
TTGTCCGGATTCGCCACAGAGAAGTTGTTCTCAAAGCCA,
CCCGGAAAGAACTTGTAAATCATCACAAACATCGCTAAGA-TGAATAAGTG
GATCATAAATGGTTCCGTGCGGGTCAGGAATTTAGAAAA-CACTAACATAT.

For gene assembly and amplification, PCR was carried out in a 50 μl mixture containing 25 μl of oligonucleotides and 25 μl of enzyme mix (FideliTaq; #71182, USB). The tube contained 1 pmol of each oligonucleotide except for the 5′-end oligonucleotides for each DNA strand. These two oligonucleotides also functioned as amplification primers and were present at 50-fold molar excess over the internal ones to achieve both assembly and amplification in a simple, one-tube protocol [34]. After an initial heating segment at 95 °C for 5 min, the reaction was cycled for 0.5 min at 94 °C, followed by "touch down" annealing [35] from 68 °C to 58 °C for 0.5 min (in 1° decrements per cycle) and concluding with a 0.5 min extension at 68 °C. When the lowest annealing temperature was reached, conventional PCR was continued for an additional 19 cycles under the last "touch down" parameters. The program ended with a final extension step at 68 °C for 10 min.

2.2. Incorporation of Kcv gene into plasmid

The full-length Kcv gene obtained by PCR (above) was purified on a 1% agarose–TBE gel and inserted directly into a TA vector (TOPO-TA; #K4500-01, Invitrogen). After verification by sequencing, the gene was liberated from the TA vector by digestion with *Nde*I and *Hind*III and ligated with pT7-SC6 that had been cut by the same enzymes to yield pT7-Kcv.

2.3. Modified Kcv genes

To purify Kcv and determine its subunit stoichiometry, DNA encoding an N-terminal His tag with an oligo-aspartate linker [36] was appended to the wild type gene. The amino acid sequence of the complete tag is: MHHHHHHDDDDDDDDSMG. This tag, which is present in pT7-SC7, lies between unique *Nde*I and *Nco*I sites (underlined) and contains the following sequence: 5′CATATG CACCAT-CACCACCATCATAATAACAATAATAACAACAATAATTCCA-TGGC. Once insertion of this N-terminal sequence onto the Kcv gene was achieved through PCR, homologous recombination of the resulting fragments was performed [37]. One of these fragments was generated using *Eco*NI-linearized pT7-SC7 as a template with (forward) 5′GACGACGATGATTCCATGGGCTTAGTGTTTTCTAAATTC-CTG and (reverse) 5′CAGAAGTGGTCCTGCAACTTTAT as primers. The other fragment used for homologous recombination was derived from *Hind*III-linearized pT7-Kcv using PCR with primers (forward) 5′ATAAAGTTGCAGGACCACTTCTG and (reverse) 5′ATAAAGTTGCAGGACCACTTCTG. The resulting tagged Kcv gene in the T7 expression vector (pT7-H6D8-Kcv) was used to generate hetero-oligomers with the wild type gene.

Later, however, expression work using *E. coli* S30 extracts showed that this tag was rapidly proteolysed, possibly inferring that acid proteases in the extract were acting on the aspartate motif. To prevent proteolysis, the aspartic acid residues of the tag were replaced with asparagines. This strategy proved successful as little or no proteolysis was observed in S30 extracts when Kcv carried a polyasparagine linker at its N-terminus. To introduce a His tag and octo-asparagine linker on Kcv, pT7-D8H6-Kcv (see above) was digested with *Nde*I and *Nco*I, and the resulting small fragment was replaced with two oligonucleotide cassettes that encode the new tag to yield pT7-H6N8-Kcv, cassette 1: 5′TATGCACCATCACCACCATCATAAT (sense), phosphorylated 5′GTTATTATGATGGTGGTGATGGTGCA (antisense); and cassette 2: 5′phosphorylated AACAATAATAACAACAATAATTC (sense), 5′CATGGAATTATTGTTGTTATTATT (antisense). All modified genes were subsequently verified by DNA sequencing.

2.4. In vitro transcription and translation

In vitro radiolabeled wild type and tagged Kcv polypeptides were synthesized by coupled IVTT in the presence of [^{35}S] methionine (10 μCi per 25 μl reaction, 1200 Ci/mmol, ICN) and rifampicin (20 μg/ml), with an *E. coli* T7 S30 extract (Promega) as previously described [21]. The DNA template (4 μl, 40 ng/μl) was incubated with IVTT components containing complete amino acid mix for 1 h at 37 °C, with a yield of about 200 ng/ml by comparison with the in vitro-synthetic α-hemolysin in earlier reports [20,21].

2.5. Hetero-tetramer formation

Early work had shown that the wild type Kcv polypeptide spontaneously assembles into an SDS-stabile oligomer during in vitro synthesis. To determine the stoichiometry of this oligomer, wild type and tagged Kcv proteins were co-synthesized and assembled in vitro, as described above, with various DNA ratios (4:0, 3:1 2:2, 1:3 and 0:4) of pT7-Kcv and pT7-H6N8-Kcv, respectively. The resulting homo- and hetero-oligomers were loaded on a 12.5% SDS–polyacrylamide gel [38] and electrophoresed. The gel was then dried and exposed to X-ray film.