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Structural Design and Characterization of a Channel-forming Peptide

Chartchai Krittanai* and Sakol Panyim

Laboratory of Molecular Biophysics, Institute of Molecular Biology and Genetics, Mahidol University, Phutthamonthon 4 Road, Salaya, Nakhonpathom, 73170 Thailand

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A 16-residue polypeptide model with the sequence acetyl-YALSLAATLLKEAASL-OH was derived by rational *de novo* peptide design. The designed sequence consists of amino acid residues with high propensity to adopt an alpha helical conformation, and sequential order was arranged to produce an amphipathic surface. The designed sequence was chemically synthesized using a solid-phase method and the polypeptide was purified by reverse-phase liquid chromatography. Molecular mass analysis by electro-spray ionization mass spectroscopy confirmed the correct designed sequence. Structural characterization by circular dichroism spectroscopy demonstrated that the peptide adopts the expected alpha helical conformation in 50% acetonitrile solution. Liposome binding assay using Small Unilamellar Vesicle (SUV) showed a marked release of entrapped glucose by interaction between the lipid membrane and the tested peptide. The channel-forming activity of the peptide was revealed by a planar lipid bilayer experiment. An analysis of the conducting current at various applied potentials suggested that the peptide forms a cationic ion channel with an intrinsic conductance of 188 pS. These results demonstrate that a simple rational *de novo* design can be successfully employed to create short peptides with desired structures and functions.

Keywords: Channel-forming peptide, *De novo* design, Peptide structure and function, Planar lipid bilayer

Introduction

The *de novo* design of peptides and proteins offer an attractive approach for studying protein structure and function. The general concept of *de novo* design involves the planning and construction of a protein with expectation of folding the linear sequence into a defined three dimensional structure. It has been demonstrated by an increasing number of studies that successful design leads led to the production of proteins and peptides with precise structures and functions (Lee *et al.*, 1997; Wierzbicki *et al.*, 2000; Lear *et al.*, 2001; Schnepf *et al.*, 2001; Wei *et al.*, 2003). Over the past few years, studies and characterizations of protein structures, especially those with a helical conformation have significantly improved our understanding of how secondary structures are formed and stabilized (Gromiha and Selvaraj, 2001; Kimura *et al*., 2002). The accumulated knowledge of theoretical and experimental studies is being applied to construct proteins with custom structures and specific functions.

Our experiences of the constructions and characterizations of trans-membrane helical peptides has revealed that the design of helical structures involves not only the selection of amino acid residues according to their helical propensity but also their associations with several other factors. These factors include local interactions and stabilizations between, neighboring residues (Stellwagen *et al*., 1992; Olson *et al.*, 2001), buffers and solvent environments (Krittanai *et al*., 2000), and considerations of conformational entropy (Aurora *et al*., 1997).

This work aimed to demonstrate the process of helical peptide design based on a basic knowledge of protein secondary structure, experimental construction, and peptide product characterization. The results obtained show that the synthesized peptide not only adopts the helical conformation but also conducts ions across an artificial membrane. The study provides an example of the role of *de novo* design in peptide structural and functional engineering.

Materials and Methods

Sequence design and structural modeling Amino acids were selected based on their helical propensities (Li and Deber, 1994; Myers *et al*., 1997). The residues used were alanine (A), leucine (L), lysine (K), glutamate (E), serine (S) and threonine (T). The

*To whom correspondence should be addressed.
Tel: +66-2-800-2732; Fax: +66-2-441-9906
E-mail: stckt@mahidol.ac.th

sequential order YALSLAATLLKEAASL was assigned to generate an amphipathic helix containing a hydrophobic and a hydrophilic region. The aromatic tyrosine (Y) residue was linked into the amino terminal of the sequence to assist in concentration determination by UV absorption (Edelhoch, 1968; Pace *et al*., 1995). The three-dimensional structure and conformational energy calculations of the designed sequence were analyzed on a Silicon Graphics workstation (SGI, USA) using Insight II software package (Molecular Simulation Inc., USA). The structural model was initially built from standard alpha helix parameters and then minimized for conformational energy using the steepest descent algorithm.

Peptide synthesis and purification Solid phase peptide synthesis was performed using a permeable bag containing p-alkoxybenzyl alcohol resin and Fmoc amino acids. (Houghten, 1985). The Fmoc protecting group was removed from amino ends with 30% piperidine and the new residue was coupled in a mixed cocktail containing N, N-Diisopropylethylamine (DIPEA), 2-(1H-Benzotriaole-1-yl)-1,1,3,3,-Tetramethyluronium tetrafluoroborate (TBTU), and 1-Hydroxybenzotriaole hydrate (HOBT). Dimethyl formamide (DMF) was used as a solvent for all solutions and washings. The peptide product was cleaved from the solid resin using 95% trifluoroacetic acid (TFA) and precipitated with ice-cold diethyl ether. Crude peptide solubilized in 50% acetonitrile was injected into an AKTA purifier (Pharmacia, Uppsala, Sweden) equipped with a PepRPC 10/10 reverse phase column. An elution gradient from 10 to 85% of 0.1% TFA/ACN was programmed over 20 min at a 1.5 ml/min flow rate. Fractions were collected corresponding to absorption at 280 nm and lyophilized before use.

Mass spectrometry The molecular mass of the synthetic peptide was determined by electrospray ionization mass spectrometry (ESI-MS) on an API-365 LC/MS/MS triple quadrupole mass spectrometer (PE SCIEX, USA). The instrument was equipped with an atmospheric pressure ionization source. Briefly, a sample in MeOH/ ACN (1 : 1, v/v) was loaded into a 100-µl glass syringe and injected into the system using an automatic plunger at 5 µl/minute. The ionization potential used was 4800-5200 volts. The orifice potential was varied from 20-120 electron volts and the electron multiplier detector was set to 2200 eV. Q1 scan was monitored in the 200-2000 m/z region.

Determination of peptide concentration Peptide concentrations were determined from the extinction coefficient of tyrosine (1,280 cm^{-1} M^{-1}) at 280 nm (Pace *et al*., 1995). The absorption of the purified peptide in a Suprasil quartz cuvette of 0.1 cm pathlength was scanned from 260 to 320 nm on a Cary-300Bio UV-Vis spectrophotometer (Varian).

Circular dichroism spectroscopy Structural characterization was performed on a JASCO J-715 spectropolarimeter (JASCO Inc.) purged with nitrogen (15 litre/min). Samples in sodium phosphate buffer pH 7.20 and in 50% ACN were analyzed at 1 to 10 mM. CD spectra in the far UV region from 190 to 260 nm were obtained at a scanning rate of 20 nm/min with a 2 seconds response time, 2 nm bandwidth, and 5 accumulations.

Liposome binding assay Liposomes with entrapped glucose were prepared using a modification of the method described by Kinsky (1974). The lipid mixture used contained 12.5 µmol phosphatidylcholine (PC), 3.6 µmol dicetyl phosphate, and 1.8 µmol cholesterol. Solvent in the mixed lipid was removed under vacuum and the lipid film was resuspended in a 300-mM glucose solution. Small unilamellar vesicle (SUVs) was prepared by squeezing the suspension through an extruder membrane with a 0.1 µm pore diameter (Avanti Polar Lipid, USA). Excess glucose was removed using a PD-10 gel filtration column (Pharmacia). An aliquot of liposome was added into a 1-ml disposable cuvette (Brand, Germany) containing hexokinase, glucose-6-phosphate dehydrogenase, 1 mM ATP, 0.5 mM NADP, 2 mM Mg(OAc)2 in 150 mM KCl, and 10 mM HEPES, pH 8.0. Glucose release was monitored at 25°C for NADPH absorption at 340 nm using a HP 8453 UV-Vis spectrophotometer (Hewlett Packard, USA). Relative glucose-release activities were calculated as a fraction of maximum release, which was defined as the amount released after adding 0.1% Triton X-100 (Puntheeranurak *et al*., 2001).

Planar lipid bilayer experiment This experiment was performed on an Axopatch-1D patch-clamping system (Axon Instruments, Foster city, USA). A CV-4B-0.1/100U Headstage (50MΩ-50GΩ resistance) was used for voltage activation and detection on the artificial membrane. The reaction chambers were shielded in a Faraday case and grounded for noise reduction. Artificial membranes were prepared fresh for each experiment from a mixture of phosphatidyl ethanolamine (PE), phosphatidyl chlorine (PC), and cholesterol (CH) at 7 : 2 : 1 ratio. An aperture (200-µm diameter) on the Teflon reaction cup was pretreated with lipid mixture and dried under nitrogen gas purging. The cup was placed in the reaction chambers and filled with 150 mM KCl, 10 mM Tris, 1 mM $CaCl_2$, pH 8.0. After the aperture had been painted with the lipid mixture, the baseline signal was monitored for membrane stability for at least 30-45 minutes and then the current was recorded at a command potential 0.0 mV. Purified peptide (5-10 µg) was then added into the *cis* chamber and induced to incorporate into the membrane by varying the command potentials. When a square current was detected, data were recorded by applying various command potentials between –100 and +100 mV.

Results and Discussion

Design, synthesis, and purification of the peptide The design of proteins and peptides for specific functions remains a challenge. However, successes in this field prove that our accumulated knowledge of the basic principles of protein structure and function are accurate, and this offers us the opportunity to construct novel proteins. In this work, we employed a simple approach to peptide design and aimed to achieve membrane interaction and pore-formation. Our initial step was to inspect the three-dimensional structures of several naturally occurring pore-forming peptides and protein fragments. Coordinates of x-ray crystal structures from the protein data bank for melittin (Terwilliger and Eisenberg,

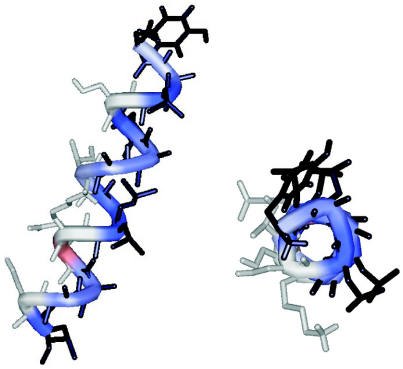

Fig. 1. Graphical representation of the model peptide, Ac-YALSLAATLLKEAASL viewed from the side (A) and the top (B). Hydrophobic and hydrophilic amino acid side-chains are colored black and gray, respectively.

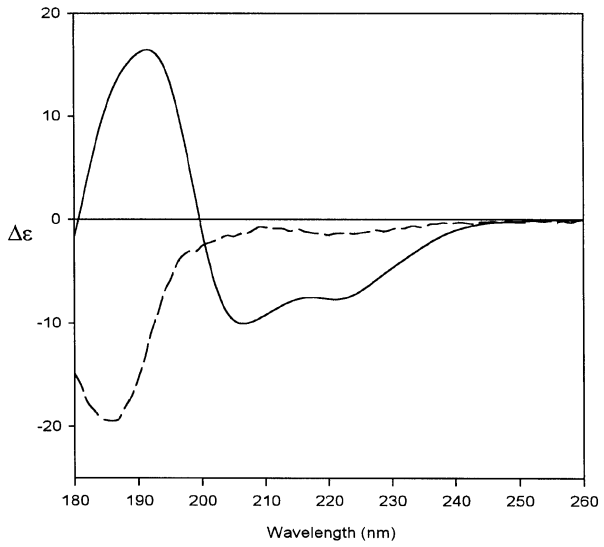

Fig. 2. Conformational analysis of Ac-YALSLAATLLKEAASL by circular dichroism spectroscopy revealed a random coil conformation (----) in 10 mM phosphate buffer pH 7.2 and a helical conformation (—) in 50% acetonitrile.

1982; PDB code-2mlt), magainin (Vogel and Jahnig, 1986; PDB code-2mag), colicin (Parker *et al*., 1992; PDB code-1col), ectatomin (Nolde *et al*., 1995; PDB code-1eci) and the pore-forming fragment of cry3a (Li *et al*., 1991; PDB code-1dlc) were examined, especially in the membrane insertion regions. A common characteristic of these pore-forming fragments is a helical peptide of 15-30 amino acids with an amphipathic surface. Our peptide sequence, acetyl-Y-ALSLAATLLKEAASL-OH was designed to conform with this common feature (Chmielewski and Lipton, 1994). The length of the synthesized peptide was set at 16 residues, based on a report of a synthetic hemolytic peptides with 12-22 residues (Cornut *et al*., 1994). A structural model of this peptide was constructed and energetically minimized (Fig. 1). The data obtained revealed a conformational stability comparable to the control conformation of natural peptides. N-terminal tyrosine was added to allow accurate peptide concentration determination and to enhance interaction with the lipid membrane. The designed peptide product was successfully obtained by solid phase synthesis and then purified on a reverse-phase column. The purified peptide fraction was collected as a large major peak based on 280-nm absorption. Elution gradient was observed around 70-80% of acetonitrile.

Sequence confirmation and conformational analysis The purified peptide was found to have the correct sequence according to a detected molecular mass ion at 1676 amu, which is identical to the molecular mass calculated for the sequence (data not shown). A structural conformation of the peptide was then characterized by circular dichroism spectroscopy. It is known that short peptides tend to adopt an unstructured random coil in an aqueous environment. However, conformation analysis of transmembrane and pore-forming peptides is generally performed in hydrophobic buffers (Gesell *et al*., 1997). We utilized an organic buffer, acetonitrile/water (1 : 1), from the purification step to mimic the hydrophobic environment of the lipid membrane. Circular dichroism spectra of the peptide in this hydrophobic buffer showed two negative bands around 207 and 222 nm and one intensely positive band at *ca.* 193 nm (Fig. 2). These spectral features were accepted to represent a conformation with a high degree of alpha helical character (Alder *et al*., 1973; Johnson, 1990). As was expected, the CD spectrum of peptide in aqueous water indicated an unstructured random coil conformation with a major negative band at *ca.* 186 nm. This conformational analysis suggested that our synthesized peptide should be able to adopt the expected alpha helical conformation in the non-aqueous environment of the lipid membrane.

Liposome binding assay To investigate the interaction between the designed peptide and the lipid membrane, a liposome assay using small unilamellar vesicle (SUV) was employed. The measurement of glucose release was based on the conversion of glucose into glucose-6-phosphate (G6P) by hexokinase, and the subsequent production of NADPH from NADP by glucose-6-phosphate dehydrogenase. The reactions took place in the solution surrounding the liposome and their progresses were detected by measuring increased NADPH absorption at 340 nm. We started the experiment by mixing enzymes and all substrates with SUVs on the 50 s time point and the peptide was then added on the 500 s progression. An initial increased absorption at *ca.* 50 s was presumed to be due to the continuous leakage of glucose from the vesicle during the test sample preparation (Fig. 3). The final full release of glucose was then accomplished upon adding Triton X-100 at *ca.* 900 s. The tested peptide was found to initiate the release of entrapped glucose after a short incubation period. The effect was

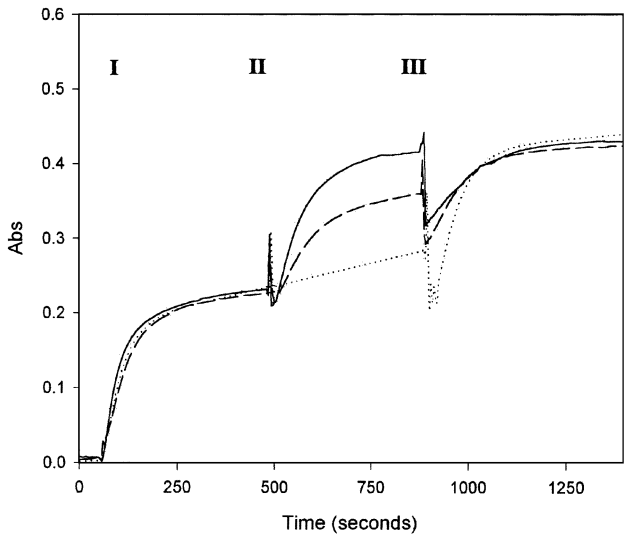

Fig. 3. Liposome binding assay using glucose-entrapped SUV showing the release of a glucose molecule upon interacting with the tested peptide, Ac-YALSLAATLLKEAASL at 0 μM (···), 10 μM (---), and 20 μM (—). Enzyme-substrate mixture, tested peptide, and triton-X were added to the liposomes at the times indicated as I, II and III, respectively.

demonstrated by a significant increase in NADPH absorption. The absorption baseline due to the buffer showed only a slow and steady change of absorption that may have been due to a slow leakage of glucose from the SUV. The activity of the peptide with SUV demonstrated concentration dependence as it showed an increased activity from approximately 50% to 90% of glucose when the peptide concentration was raised from 10 to 20 μM. The experimental assay revealed that our designed peptide could interact with the unilamelar lipid membrane and release glucose entrapped in vesicles. However, this experiment does not provide a detail mechanism for this interaction.

Ion channel activity assay using planar lipid bilayers

Further investigation of the peptide was based on its channel forming activity in a planar lipid bilayer (PLB) system. This highly sensitive technique is widely used to analyze the qualitative and quantitative parameters that describe the characteristics of protein and peptide ion channels. The experiment was performed in symmetrical mode to verify the channel activity and to determine the characteristic channel conductance. The results are shown as a number of square jumps of conducting current at various applied potentials (Fig. 4A). Even though the conducting state occupied most of the

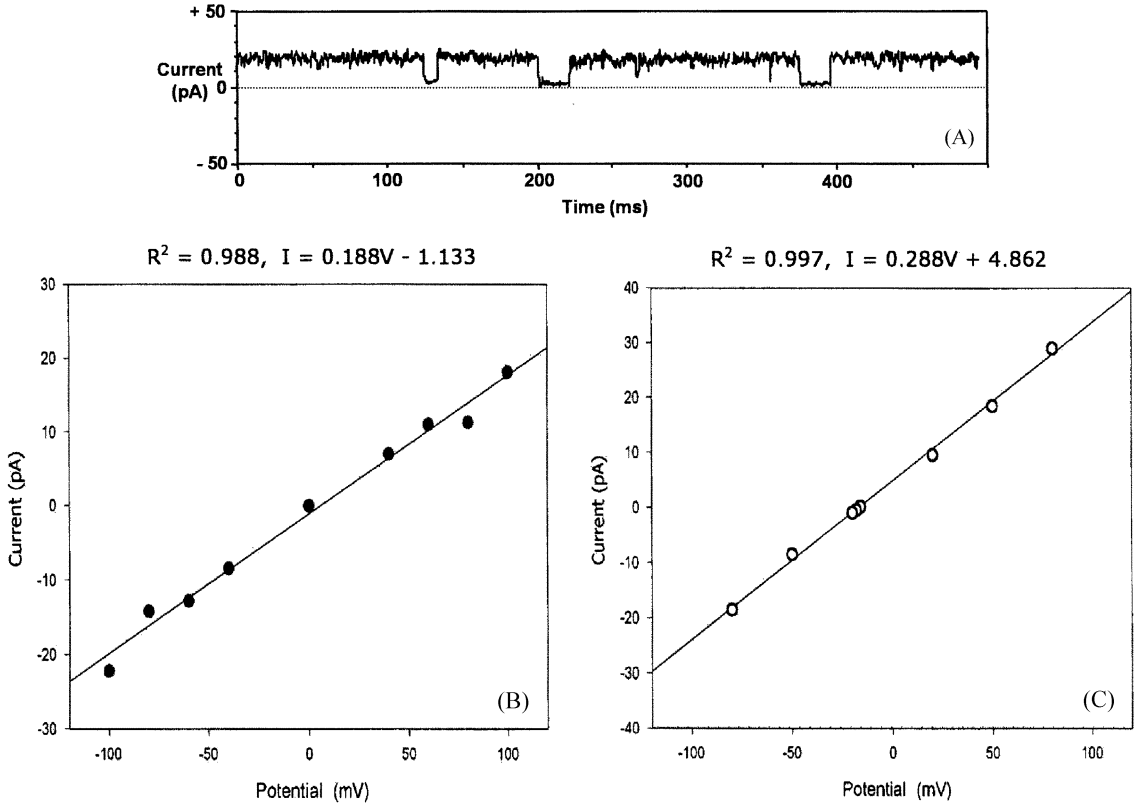

Fig. 4. The conducting current detected during the planar lipid bilayer experiment showed several square jumps at various times (A). The plot between the current and applied potential were obtained using a symmetric system (B) containing 150 mM of KCl in cis-and trans-chambers and using an asymmetric system (C) containing 450 and 150 mM KCl in the cis- and trans-chambers, respectively.

monitoring time, the non-conducting state is identified as a jump to zero current. The detected conducting currents were then plotted against the various applied potentials from –100 to +100 millivolts. The determined slope, representing channel conductance, was 188 picosiemens (pS) (Fig. 4B). This characteristic conductance is comparable to that of the channels formed by natural cytotoxic peptides like melittin and insect defensins, which fall in the range 100-200 pS (Pawlak *et al*., 1991; Cociancich *et al*., 1993).

To determine whether the channel created was cation or anion selective, an asymmetrical experiment was performed using different concentrations of KCl in the *cis* and *trans* chambers. A plot of detected currents against applied potentials was obtained with excellent linearity. Unlike the symmetrical mode, this plot (Fig. 4C) obtained from the asymmetrical experiment shows a non-zero current at zero applied potential, which means that a reverse potential is required to halt the ion flow. This reversed potential was obtained adjusting the voltage to zero current; this was found to be −16.88 mV. This negative potential indicates that the ion was due to K^+. Thus, the channel formed by this peptide can be classified as a cation-selective channel.

The model of channel formed by a short peptide like magainin was suggested to involve three channel models (Cruciani *et al*., 1992). Type I channels are solely composed of multimeric transmembrane peptides with hydrophilic faces aligned to form a hydrophilic pore, whereas the hydrophobic faces interact with the lipid. In type II and III channels the channel walls are mainly composed of lipid head groups and the peptide lining on the membrane surface to stabilize the pore opening. In the present work, the synthesized peptide may adopt either a Type II or Type III channel according to the marginal length of the peptide chain and the observed cation selective characteristics (Kourie and Shorthouse, 2000). However, elucidation of the molecular dispositions in the channel is not straightforward, and is known to depend on a number of factors including lipid composition and peptide charge.

Conclusion

This study demonstrates that the straightforward process of constructing *de novo* peptides can be applied to produce a short alpha-helical peptide, which functions by interacting to form an ion channel in a lipid membrane. The design strategy was based on the selection of amino acid residues with high helical propensities and their sequential alignment to form an amphipathic surface. The designed sequence was chemically synthesized, purified, and characterized structurally and functionally, and shown to be an amphipathic peptide with a helical conformation that interacts with lipid and releases glucose from liposomes. Its channel activity showed that it forms a cation selective pore with a characteristic conductance of 188 pS.

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