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Chemical Derivatization and Purification of Peptide-Toxins for Probing Ion Channel Complexes

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Abstract

Ion channels function as multi-protein complexes made up of ion-conducting α -subunits and regulatory β -subunits. To detect, identify, and quantitate the regulatory β -subunits in functioning K^+ channel complexes, we have chemically-derivatized peptide-toxins that specifically react with strategically-placed cysteine residues in the channel complex. Two protein labeling approaches have been developed to derivatize the peptide-toxin, charybdotoxin, with hydrophilic and hydrophobic bismaleimides, and other molecular probes. Using these cysteine-reactive peptide-toxins, we have specifically targeted KCNQ1-KCNE1 K^+ channel complexes expressed in both *Xenopus* oocytes and mammalian cells. The modular design of the reagents should permit this approach to be applied to the many ion channel complexes involved in electrical excitability as well as salt and water homeostasis.

Keywords

ion channel; regulatory subunit; scorpion toxin; electrophysiology

1. Introduction

Ion channels co-assemble with different regulatory β -subunits to afford membrane-embedded complexes with diverse ion-conducting and voltage-gating properties that fulfill the ion permeation needs of a wide range of cells and tissues (1-4). Ion channel complex assembly is vital for proper cellular function, as mutations that prevent proper complex formation give rise to neurological, cardiac, and muscular diseases (5-7). While the importance of these membrane-embedded complexes is widely appreciated, it remains challenging to detect, identify, and quantitate the regulatory subunits in functioning ion channel complexes.

To overcome some of these challenges, we have chemically-derivatized peptide toxins to specifically react with target cysteine residues placed in the regulatory β -subunits of functioning K^+ channel-KCNE complexes (8, 9). The peptide-toxin is derivatized on the non-binding side of the toxin with a bismaleimide linker that converts these reversible inhibitors into cysteine-reactive reagents. Application of a low concentration ($\sim$ nM) of the derivatized toxin to cells expressing the K^+ channel complex results in first binding to the

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complex (Fig. 1A) followed by a rapid and irreversible reaction with an appropriately-positioned cysteine residue in the KCNE regulatory subunit. The specificity and rapid labeling of the regulatory subunit arises from the toxin's avidity for the ion-conducting subunit, which increases the local concentration of the cysteine-modifying group (maleimide) near the target cysteine in the ion channel complex. Without this toxin-aided increase in effective concentration, the bimolecular reaction between the maleimide and cysteine would not measurably occur. Toxin binding and chemical modification are monitored by measuring the current flowing through the K⁺ channel complexes since toxin binding—both reversible and irreversible—completely blocks ion permeation.

The approach has several technical and experimental advantages: (i) Peptide-toxins can be readily expressed as fusion proteins in *E. coli* and the proteolytically-cleaved, fully-folded toxin can be purified by reverse-phase HPLC. Alternatively, peptide-toxins can be synthesized by solid-phase peptide synthesis, refolded and similarly purified by HPLC. (ii) The folded toxins are very stable and compatible with organic solvents, enabling the chemical derivatization of the toxin with hydrophobic compounds, linkers and probes. (iii) The chemically-derivatized toxins are extremely water soluble, reducing the non-specific binding observed with many hydrophobic reagents and probes. (iv) Ion channels where no known toxin exists can be mutated to bind a particular toxin with high affinity. We have previously exploited this approach to specifically target exogenously-expressed KCNQ1 K⁺ channel complexes in *Xenopus* oocytes (8, 9).

The following procedures detail two approaches to label the peptide-toxin, charybdotoxin, with a hydrophobic, reductant-cleavable linker (Fig. 1B, CTX-Clv) and with a water-soluble, non-cleavable linker (Fig. 1B, CTX-Mal). The modularity of the approach combined with the assortment of peptide-toxins available to specifically inhibit different classes of ion channels should allow this approach to be readily applied to a variety of membrane-embedded ion channel complexes.

2. Materials

2.1. Bismaleimide Modification of Peptide-Toxins Containing a Modifiable Cysteine Residue

1. Recombinant charybdotoxin, CTX-R19C, was purified as the methanethiosulfonate ethyltrimethylammonium (MTSET)-protected disulfide, as described by Shimony and Miller (10).
2. DL-dithiothreitol (DTT, for molecular biology, Sigma-Aldrich) is dissolved at 1 M in deionized water and stored in single use (1.5 mL) aliquots at -20°C.
3. Bismaleimides (Pierce, other vendors or home-made)
4. Organic co-solvent for dissolving hydrophobic bismaleimides (dimethylformamide (DMF) and/or acetonitrile (ACN))
5. Potassium Phosphate 1 M, pH 7.1

2.2. Sulfopropyl-Sephadex (SPS) Separation of Modified Toxins

1. Sulfopropyl-sephadex resin (SPS, Sigma-Aldrich# SPC25120, dry bead 40-125 μ)
2. Solution of 1 mM ethylenediaminetetraacetic acid (EDTA, Fisher), pH 7.1
3. SPS Buffer C (low salt): 10 mM KCl, 10 mM potassium phosphate at pH 7.4, 7.1 and 6.0
4. SPS Buffer D (high salt): 1 M KCl, 10 mM potassium phosphate, pH 6.0

5. Bio-Rad glass “Econo-Column” column (1 × 10 cm)

2.3. Reverse Phase HPLC Purification of Modified Toxins

1. Solvent A (aqueous): 0.1% trifluoroacetic acid (TFA, Sigma-Aldrich) in deionized water
2. Solvent B (organic): acetonitrile (ACN, HPLC-grade, Fisher)
3. Analytical C18 HPLC column (Protein and Peptide C18, 5 μ m, 4.6 × 250 mm, Vydac)
4. Large volume injection loop (5 mL)
5. Organic solvent compatible 0.45 μ m syringe filters (Life Sciences, HPLC certified)

2.4. Modification of K⁺ Channel Complexes with Maleimido-Toxins

1. Reduced glutathione (GSH, Sigma-Aldrich# G4251)
2. Bovine Serum Albumin (BSA, Sigma-Aldrich# A3059)
3. Peristaltic pump (optional)
4. External recording solution for either *Xenopus* oocytes or mammalian cells

3. Methods

3.1. Labeling and Purification of CTX-MTSET with a hydrophobic bismaleimide

1. CTX-MTSET (16 nmol) is dissolved in 2 mL of SPS Buffer C at **pH 7.4** (Note 4.1.1).
2. The free thiol of CTX-R19C is generated by reduction with DTT (2 μ L of a 1 M stock solution) for 45 min (Note 4.1.2).
3. The reaction mixture containing reduced CTX-R19C is directly injected onto an analytical C18 HPLC column that is pre-equilibrated in Solvent A: 95%; Solvent B: 5% and eluted with a gradient of 5 – 40 % B over 35 min (Note 4.1.3). Exact elution gradient is shown in Fig. 3 (Note 4.1.4). Toxin signal (underivatized and derivatized) is best detected by monitoring at 280 nm.
4. The peak containing the DTT-free, reduced CTX-R19C is collected (Fig. 3A) and the solution is adjusted to pH 7.0 with 1M potassium phosphate, **pH 7.1** (Note 4.1.5).
5. The solution of neutralized, reduced CTX-R19C is slowly added to a solution of 16 μ mol of bismaleimide in 100 μ L of an organic solvent (ACN, DMF) with vigorous swirling and allowed to react for 30 min at room temperature (Notes 4.1.6 and 4.1.7).
6. The reaction mixture is placed on ice for 10 min to precipitate excess unreacted bismaleimide, which is removed by filtration using an organic solvent compatible syringe filter (GHP Acrodisc 0.45 μ m, Pall Gelman Laboratory).
7. The mono-derivatized CTX-R19C is HPLC-purified using a 20 – 50 % B gradient over 30 min (Fig. 3B).
8. The peak corresponding to the mono-derivatized CTX-R19C (CTX-Clv) is collected, the majority of which is immediately aliquoted into microcentrifuge tubes (30 – 50 μ L aliquots), flash frozen with liquid nitrogen, lyophilized to dryness without heating, and stored at – 20°C (Note 4.1.8).

9. A small sample (75 – 100 μ L) is used to both determine the concentration of the aliquots and to determine the molecular weight of the derivatized toxin.
10. To determine the concentration of the aliquots, a quartz UV cell is blanked on the HPLC elution buffer based on the approximate Solvent B % of the collected peak. For example in Fig. 3C, a mixture of 75% Solvent A and 25% Solvent B was used as the blank. Under these conditions, 1 OD @ A_{280} = 1 mg/mL for CTX (Note 4.1.9). Overall yield of the purified maleimide-toxins are ~ 20 – 40%.
11. For mass spectrometry, the collected sample is directly infused into an electrospray ionization (ESI) mass spectrometer in the positive mode (Fig. 4). As expected for a protein with many arginine and lysine residues, multiply-charged species are observed in the mass spectrum. Since the toxins are HPLC-purified with 0.1% TFA, TFA-adducts are also observed in the mass spectra (Note 4.1.10).

3.2. Labeling and Purification of CTX-MTSET with a hydrophilic bismaleimide

1. CTX-MTSET (10 nmol) in 0.5 mL of SPS Buffer C at **pH 7.4** is reduced by adding 1 μ L of 0.5 M DTT (Note 4.1.5).
2. After 30 min, the reaction is diluted with 5 mL of 1 mM EDTA, **pH 7.4** and is applied to a SPS column (0.3 g) equilibrated in SPS Buffer C.
3. The excess DTT is washed out with 60 mL of SPS Buffer C, **pH 7.1** (slight pressure can be applied with a peristaltic pump or hand-held motorized pipetting device).
4. Dissolve bismaleimide (10 μ mol) in 1 mL SPS Buffer C, **pH 7.1** (Note 4.1.6). For bismaleimides with intermediate hydrophobicity, ~100 μ L of an organic co-solvent can be used to first dissolve the bismaleimide. The bismaleimide solution can contain up to ~ 10% organic solvent (Note 4.1.7).
5. Slowly apply the 10 mM bismaleimide solution to the top of column and allow the entire solution to penetrate the SPS resin. Cap the bottom of the column (to prevent leakage) and incubate for 15 min.
6. Wash out excess linker with 50 mL of SPS Buffer C, **pH 6.0**. (Precious, in-house synthesized or expensive bismaleimides can be partially recovered by collecting the first 20 mL of the eluent and purifying it in 4 mL fractions by reverse phase HPLC) (Note 4.1.3).
7. Elute the mono-derivatized toxin with SPS Buffer D, **pH 6.0**; collect 4 $\times$ 4 mL fractions.
8. The four fractions are individually desalted and HPLC-purified on an analytical reverse phase C18 column using the 5 – 40% B gradient (35 min) in Fig. 3C. Fractions 1 and 2 are usually the most concentrated fractions (Note 4.1.3).
9. The peak corresponding to the mono-derivatized CTX-R19C is collected, aliquoted, quantitated, characterized and stored as described in *Method 3.1*.

3.3. Application of CTX-maleimides to cells expressing cysteine-containing K⁺ channel complexes

1. Mammalian cells or *Xenopus* oocytes expressing ion channel complexes harboring extracellular cysteine residues should be incubated in 2 mM reduced glutathione to prevent cysteine oxidation that cannot be reversed with DTT or TCEP. The media should be changed daily to maintain an extracellular reducing

environment (Note 4.1.11). Cells can be incubated without glutathione; however, the labeling efficiency will be compromised.

- 2 The cells are first treated with 2 mM TCEP (or DTT) in external recording solution for 5 min to fully reduce the extracellular cysteines on the cell surface.
- 3 The TCEP-treated cells are rinsed, voltage-clamped and the external recording solution should contain 50 μ g/mL BSA to prevent the toxins from non-specifically binding to the recording chamber, tubing, etc.
- 4a The CTX-maleimides are dissolved in the appropriate electrophysiological recording solution (containing BSA) and are immediately perfused into the recording chamber. For CTX-sensitive KCNQ1-KCNE1 complexes, we have used 10 nM CTX-maleimide to achieve > 90% inhibition. The pH of the recording solution should be between 7.0 and 7.6 for optimal labeling (Note 4.1.5).
- 4b For the more hydrophobic CTX-maleimides, the toxin is first dissolved in a minimal amount of 30% ACN in external recording solution and then diluted with external recording solution such that the final toxin solution contains less than 0.3% ACN.
- 5 If continuous perfusion is required for stable current measurements, a peristaltic pump can be used to recycle the toxin solution back into the recording chamber.
- 6 The cells are treated with CTX-maleimide for 5 min before the excess reagent is washed out. For optimally placed cysteine residues, the reaction is usually complete within 100 – 150 sec (Notes 4.2.1 and 4.2.5).
- 7 After the excess toxin is washed out, the extent of labeling is determined by the amount of irreversible inhibition (Fig. 5A).
- 8 For CTX-maleimides with chemically-cleavable linkers, the reagent to cleave the linker (in this case TCEP) is perfused into the bath using the BSA-containing recording solution (Fig. 5B). The cleavage reaction is monitored as a return of current to pre-treatment levels.

4. Notes

4.1. General Notes

1. CTX-R19C is purified as the mixed disulfide (CTX-MTSET) to prevent both toxin dimerization (via disulfide bond formation) and cysteine oxidation. In addition, the mixed disulfide is required to cyclize the N-terminus of the toxin (10).
2. DTT reduction of CTX-MTSET should not last longer than 60 min since longer incubation times (as well as DTT concentrations > 1 mM) leads to reduction of the internal disulfide bonds of the toxin. Accordingly, the stronger reducing agent, TCEP, should not be used because it rapidly reduces and induces unfolding of the toxin.
3. A 5 mL injection loop is essential for rapid purification of the derivatized toxins due to the milliliter volumes needed for the SPS column separation and chemical reactions. In addition, we never inject more than 4.5 mL since we have noticed that some of the sample exits the loop no matter how slowly/gently the sample is loaded with volumes greater than 4.5 mL.

4. Much of the work of the HPLC is to remove the excess salts from the SPS Buffers or to remove DTT; therefore, our standard gradient involves an initial isocratic segment for 10 – 15 min (Fig. 3), which removes the majority of the side products.
5. The pH of the solutions for the cysteine modifications and maleimide reactions are critical for obtaining toxins with an intact, reactive maleimides. Thus, we have emboldened the pH of the solutions since it changes during the preparation of the derivatized toxins. In general, thiol-modifying reagents require a pH > 7.0, which generates a significant concentration of the reactive thiolate anion. However, once the reactions are complete, the pH is lowered to ~ 6.0, which reduces the hydrolysis of the remaining intact maleimide.
6. The organic co-solvents will need to be optimized for each individual bismaleimide. The major complication is due to the bismaleimide precipitating out when the toxin is added to the bismaleimide solution. The reverse addition—adding the bismaleimide in neat organic solvent to the toxin in a water/organic mixture—always resulted in precipitation in our hands.
7. The concentration of the maleimide is critical for efficient labeling. It is preferable to operate at least in the single-digit mM range, ensuring a rapid reaction between thiol and maleimide while minimizing maleimide hydrolysis. Since bismaleimides can potentially react with two CTX-R19C toxins, a 100 – 1000-fold molar excess of bismaleimide (compared to toxin) is used to eliminate this potential side reaction.
8. Although we rapidly freeze dry the HPLC-collected samples, the CTX-maleimides and bismaleimides are reasonably stable (~ hrs) in HPLC solvents that contain TFA.
9. The classic ninhydrin assay (11) can be used as an alternative to the “quick and dirty” UV spec method for determining derivatized toxin concentration. We also always compare the apparent binding affinity of the newly synthesized derivatized toxin to underivatized toxin (See Note 4.2.4).
10. For CTX-R19C, the lone methionine residue in the toxin is often oxidized, which shifts the molecular weight of the toxin by 16 Daltons.
11. We have consistently made the glutathione-containing media fresh each day from powder; however, similar to DTT, concentrated glutathione aliquots should be stable at – 20°C.

4.2. Development Notes

The following describes the series of steps taken to develop a new ion channel-derivatized toxin pair.

1. The exact location of the target cysteine in the ion channel complex is critical for a rapid and quantitative reaction (> 99%). Thus, it is prudent to make several single-cysteine mutations in the region of interest in the ion channel complex.
2. Linker length of the bismaleimide is the second variable that needs to be optimized. We have found that a ~ 20 Å linker is sufficient for labeling the external face of the pore-forming unit (S5-P-S6) of voltage-gated K⁺ channels; a ~ 40 Å linker seems sufficient to reach most extracellular regions of the voltage-sensing unit (S1-S4), though we have been able to label the extended S3-S4 loop of the *Shaker* voltage sensor with the shorter, 20 Å linker.

3. Identifying the maleimido-toxin in the HPLC chromatogram can be challenging, in particular, if *Method 3.1* is used. During the development phase, we collect each peak in the chromatogram and identify the peaks using ESI mass spectrometry. The positively-charged toxins ionize very well, providing strong, easily identifiable signals, even in the presence of the ion-suppressing TFA.
4. Once purified, first test the maleimido-toxin on the “wild-type” construct to ensure that the toxin does not react with any of the endogenous cysteines in the ion channel complex as in Fig. 5C). If irreversibility is observed, a systematic removal of the cysteines in the external half of the ion channel complex is needed. If complete reversibility is observed, these experiments also serve to characterize the derivatized toxin’s binding affinity, and off- and on-rates to ensure that the chemical tinkering did not significantly perturb toxin binding to the ion channel complex.
5. Second, test the derivatized toxin on the different single-cysteine subunits. We have found the rate and the extent of reaction can vary significantly (40 – 100%) on the location of the target cysteine. We have not tested whether this variation is due to steric hindrance or orientation of the target cysteine.
6. Lastly, ensure that the irreversible labeling observed is dependent on toxin binding by performing a competition experiment with unlabeled toxin (Fig. 5D). Successful prevention of labeling with excess competitor demonstrates that the non-specific, bimolecular reaction between the maleimido-toxin and the ion channel complex is not appreciably occurring.
7. If no peptide-toxins are known for a particular ion channel complex, toxin binding-site transplantation is an option. For K⁺ channels, several transplants have been successfully documented (12-16).

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Fig. 1.

(A) Cartoon depiction of the derivatized peptide-toxin (CTX) binding to the K⁺ conducting subunit followed by the tethering reaction between the maleimide and cysteine residue in the regulatory β -subunit. (B) Molecular structures of the derivatized peptide-toxins: CTX-Clv and CTX-Mal. Scissors indicate the TCEP cleavable bond in CTX-Clv.

Fig. 2. Two methods for labeling the peptide-toxin, CTX, with hydrophobic and hydrophilic bismaleimides.

Fig. 3. HPLC traces of reduced CTX-R19C and bismaleimide-labeled CTX-adducts. Absorbance signal is measured at 280 nm (left axis). Dashed lines indicate the solvent gradient in %B (right axis). The collected peaks are bracketed between t1 and t2.

Fig. 4.

Electrospray mass spectra of purified CTX-Clv and CTX-Mal in the positive mode. The different charge species for the derivatized toxins are denoted in brackets. Expected MW for CTX-Clv: 4933 (methionine oxidized); +3H, 1,646; +4H, 1,234; +5H, 988. Expected MW for CTX-Mal: 4593 (methionine oxidized); +3H, 1532; +4H, 1149; +5H, 920; +6H, 767.

Fig. 5.

Utilization of CTX-Mal and CTX-Clv for labeling and identifying KCNE regulatory subunits in functioning KCNQ1-KCNE1 K⁺ channel complexes. (A) Single cysteine containing Q1/E1_{T14C} complexes were irreversibly inhibited by 10 nM CTX-Mal and did not reverse upon removal (Wash) of excess reagent. (B) Q1/E1_{T14C} complexes were first irreversibly inhibited by 10 nM CTX-Clv, which was reversed by TCEP cleavage (1 mM) of the linker connecting the maleimide to the toxin. (C) Q1/E1 complexes lacking extracellular cysteine residues are only reversibly inhibited by 10 nM CTX-Mal, demonstrating the requirement for a target cysteine residue. (D) Irreversible inhibition of Q1/E1_{T14C} by CTX-Clv requires binding to the ion-conducting subunit since excess (500 nM) competitive inhibitor (CTX unlabeled) completely prevents chemical labeling and irreversibility.