Charybdotoxin is a high-affinity specific inhibitor of the high-conductance Ca\(^{2+}\)-activated K\(^+\) channel found in the plasma membranes of many vertebrate cell types. Using Ca\(^{2+}\)-activated K\(^+\) channels reconstituted into planar lipid bilayer membranes as an assay, we have purified the toxin from the venom of the scorpion *Leiurus quinquestriatus* by a two-step procedure involving chromatofocusing on SP-Sephadex, followed by reversed-phase high-performance liquid chromatography. Charybdotoxin is shown to be a highly basic protein with a mass of 10 kDa. Under our standard assay conditions, the purified toxin inhibits the Ca\(^{2+}\)-activated K\(^+\) channel with an apparent dissociation constant of 3.5 nM. The protein is unusually stable, with inhibitory potency being insensitive to boiling or exposure to organic solvents. The toxin's activity is sensitive to chymotrypsin treatment and to acylation or glycosylation.

**MATERIALS AND METHODS**

**Fractionation of Scorpion Venom**

Venom of *L. quinquestriatus* was obtained as a lyophilized powder from Latoxan Scorpion Farm, Rosans, France, and was stored at -20 °C. A 200-ng sample of venom was dissolved in 36 ml of buffer A (40 mM NaCl, 10 mM sodium borate, 10 mM Na\(_2\)CO\(_3\), pH 9.0), and the undissolved mucoid material centrifuged at 5000 g for 10 min. The supernatant was saved and the pellet extracted twice in about 10 ml of buffer A. The combined supernatant was loaded onto a 5-ml column of SP-Sephadex equilibrated with buffer A. The column was then washed with 35 ml of buffer A until the A\(_280\) of the eluate fell to below 0.08. A linear gradient (80 ml total volume) of buffer A to buffer B (20 mM NaCl, 10 mM Na\(_2\)CO\(_3\), 10 mM Na\(_2\)PO\(_4\), pH 12.0) was then applied to the column, and the protein concentration and pH of the eluant followed. The peak of CTX activity was found to elute at pH 10.8-11.0.

Active fractions from the SP-Sephadex column were neutralized by addition of acetic acid, and were further fractionated by reversed-phase HPLC, using a 150-A pore C8 column. Sample (about 200 µg of protein) was applied to the column, which was then washed with 5 ml of buffer C (41 mM acetic acid, 3 mM sodium acetate). A linear gradient (15 ml total volume) of buffer C to buffer D (30 mM acetic acid in 50% methanol) was run at 1 ml/min, and A\(_280\) was monitored. The major CTX peak eluting at 25–30% methanol was in most cases repurified by a second pass through the reversed-phase column. Purified CTX was neutralized with Na\(_2\)HPO\(_4\), and methanol was removed by evaporation under a stream of N\(_2\) gas. The stock solution of CTX was stored frozen at -20 °C in glass tubes.

**Assay of CTX**

Activity of CTX was assayed by the ability of this protein to block the Ca\(^{2+}\)-activated K\(^+\) channel of skeletal muscle (7). Single Ca\(^{2+}\)-activated K\(^+\) channels from rat skeletal muscle transverse tubule membranes prepared as described (15) were incorporated into planar lipid bilayers formed from solutions of 21 mM 1-palmitoyl, 2-oleoyl phosphatidylcholine, 6 mM 1-palmitoyl, 2-oleoyl phosphatidylcholine (Avanti Polar Lipids, Birmingham, AL). Planar bilayers were formed with 150 mM KCl, 10 mM MOPS, 50 mM CaCl\(_2\), 5 mM KOH as the "internal" solution, and 10 mM MOPS, 0.2 mM EGTA, 5 mM KOH as the "external" solution. Transverse tubule vesicles (5 µg/ml) were added to the internal solution with stirring, and transbilayer current was measured at a holding voltage in the range 0–35 mV (external solution ground). As soon as a single channel inserted into the bilayer, as shown by the appearance of unitary fluctuations in current of about 15 pA, further insertion was suppressed by adding...
of channels was assayed in planar bilayers as described above. Con-
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Purification—We have developed a convenient two-step method for purification of CTX from the crude scorpion venom. We first exploit the unusually basic nature of the protein by employing SP-Sephadex cation exchange chromatography and pH gradient elution. A final purification by reversed-phase HPLC is then employed. Both of these steps can be used because of the exceptional chemical stability of CTX, which may be exposed to extreme ranges of pH and to solvents without loss of activity, as will be documented below.

Fig. 2A shows the protein elution profile from an SP-Sephadex column run in a chromatofocusing mode, i.e. at low ionic strength and increasing pH. Under relatively basic conditions (pH 9.0), over 90% of the protein in the crude venom runs through the cation exchange column. The protein which remains on the column is then eluted by steadily increasing the pH of the eluate, and hence neutralizing the proteins according to their isoelectric points. Channel-blocking activity is found only in the last protein peak, which elutes at pH 10.8–11.0. This is consistent with the fact that purified CTX runs off an isoelectric focusing gel utilizing a pH gradient of 8.0–10.8 (data not shown), indicating that the isoelectric point of this protein is greater than 10.8.

The SP-Sephadex fractions containing CTX activity are applied to a reversed-phase C8 column (Fig. 2B). The inhibitor is eluted as the major protein peak at about 25% methanol. Two minor peaks can also be seen, one just leading and one just lagging the major peak; these two minor peaks can be entirely removed by collecting only the central part of the
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Fig. 2. Purification of CTX. 200 mg of crude venom, dry weight, were extracted as described. A, SP-Sephadex cation exchange column (1 x 5 cm) was run in chromatofocusing mode. 0.5-ml fractions were collected. Starred fractions contain channel-blocking activity. B, starred fractions from the SP-Sephadex column of A were pooled and chromatographed by reversed-phase HPLC as described under "Materials and Methods." All CTX activity migrated in the major protein peak.

Table I
Charybdotoxin purification

| Sample          | Total protein (mg) | Specific activity (ml/mg) | Total activity (ml) |
|-----------------|--------------------|--------------------------|---------------------|
| Crude venom     | 99                 | 29 ± 3                   | 2900 (100%)         |
| SP-Sepahdex     | 1.2                | 2100 ± 200               | 2600 (90%)          |
| C8              | 0.30               | 7000 ± 1400              | 2300 (80%)          |

The efficiency of this purification scheme is summarized in Table I. The chromatofocusing step recovers 90% of the total blocking activity, and the complete procedure recovers 80% of the total activity, with an approximately 300-fold purification over the water-extractable part of crude venom. We have performed this procedure numerous times on several different batches of L. quinquestriatus venom, and have found both the yield and purification profile to be reproducible. Given the extinction coefficient and molecular mass of CTX (see below), we can estimate that this protein accounts for about 0.13% of the crude scorpion venom, and that about 1 mg of pure CTX can be recovered from 1 g of dried venom.

Previous work (7) suggested that CTX is a low molecular mass protein. We examined the mobility of purified CTX and other venom protein fractions with SDS-polyacrylamide gel electrophoresis, using highly cross-linked gels containing 6 M urea (16). The purification of CTX can be visualized on these gels (Fig. 3) with the pure product showing only a single band, even on highly overloaded gels. Reducing gels give an apparent molecular mass for CTX of about 6 kDa. We also show the protein mobility pattern on nonreducing SDS/urea gels; again, the purified material moves as a single band, and the blocking activity is preserved after cutting this band out of the gel and eluting the protein from the polyacrylamide matrix, as is shown in the figure.

Additional evidence of protein purity is provided by N-terminal amino acid analysis. After reacting the protein with dansyl chloride at pH 7.5 and hydrolyzing it, we detect only a single dansylated residue by thin-layer chromatography on polyamide plates (21). This result indicates the presence of a single species of N terminus in the protein sample. By using three different solvent systems (1.5% formic acid; benzene/acetamide, 9:1; and ethylacetate/acetamide/methanol, 20:1:1), we unambiguously identified the N terminus as valine (data not shown). This result is consistent with a preliminary sequence analysis (carried out by Dr. Clive Slaughter, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), in which valine was identified in the first cycle of Edman degradations.

Amino Acid Analysis and Determination of Molecular Mass—We analyzed the amino acid composition of the purified CTX sample following hydrolysis in 6 N HCl, under conditions where amino acid degradation is minimized. We find (Table II) that most amino acids appear in integral multiples of alanine. This result further validates the purity of the sample, and it allows us to estimate a minimum molecular mass. Assuming that CTX contains a single alanine residue, the molecular mass is 9.2 kDa.

Amino acid analysis also allows us to determine the molar extinction coefficient of CTX, again based on the assumption that the protein contains a single alanine residue. By measu-
under "Materials and Methods." Amino acids are reported as concentrations in the original sample volume. This composition gives a molecular mass of 9.2 kDa and molar extinction coefficient (280 nm) of 17,500 M⁻¹ cm⁻¹ in solution, and has been shown to be invariant for proteins of widely differing amino acid compositions. In Table III, determination of molecular mass and extinction coefficients of CTX

| Amino acid | µM | [Amino acid]/[Ala] | Assumed composition |
|------------|----|-------------------|--------------------|
| Aax        | 103| 5.3               | 5                  |
| Thr        | 140| 7.2               | 7                  |
| Ser        | 135| 6.9               | 7                  |
| Glx        | 101| 5.2               | 5                  |
| Pro        | 153| 7.8               | 8                  |
| Gly        | 93 | 4.7               | 6                  |
| Ala        | 20 | 1.0               | 1                  |
| Cys*       | 5.9| 6                 |
| Val        | 78 | 3.0               | 4                  |
| Met        | 16 | 0.8               | 1                  |
| Ile        | <5 | <0.3              | 0                  |
| Leu        | 38 | 1.9               | 2                  |
| Tyr        | 39 | 2.0               | 2                  |
| Phe        | 49 | 2.5               | 3                  |
| Lys        | 155| 8.1               | 8                  |
| His        | 44 | 2.2               | 2                  |
| Arg        | 115| 5.9               | 6                  |
| Trp        | 34 | 1.8               | 2                  |

* Determined in a separate experiment by performic acid oxidation as described under "Materials and Methods."

A sample of CTX, 140 µl of A₂₈₀ = 0.35, was hydrolyzed as described under "Materials and Methods." Amino acids are reported as concentrations in the original sample volume. This composition gives a molecular mass of 9.2 kDa and molar extinction coefficient (280 nm) of 17,500 M⁻¹ cm⁻¹ in solution, and has been shown to be invariant for proteins of widely differing amino acid compositions. In Table III, determination of molecular mass and extinction coefficients of CTX

| Method                  | Molecular mass | ε₀ | ε′ |
|-------------------------|----------------|----|----|
| Amino acid analysis     | 9.3            | 17,500 | 1.5 |
| Gel electrophoresis     | 6              |      |    |
| Mass extinction         | 11.5           |      |    |

A final method for determining the molecular mass of CTX is to combine the above value of molar extinction coefficient with an independently measured value of mass-based extinction coefficient. Molecular mass is given simply as the ratio of these two extinction coefficients. The mass-based extinction coefficient can be measured within 15% from the absorbance at 205 nm, corrected for the absorbance at 280 nm (22); this gives an empirical measure of the concentration of peptide bonds in solution, and has been shown to be invariant for proteins of widely differing amino acid compositions. In Table III, we estimate a molecular mass for CTX of 11.5 kDa using this method. Table III also summarizes our three independent determinations of molecular mass.

Properties of Purified Charybdotoxin—Charybdotoxin is an exceptionally stable protein, whose activity is maintained under conditions considered harsh for most proteins. In Table IV we demonstrate that CTX activity is present after boiling and after overnight incubation at pH 2, at pH 12, in 0.1 M SDS and 6 M urea, or in 50% methanol. It is this stability which enables us to purify the protein so straightforwardly. The activity is totally lost upon chymotrypsin treatment, by boiling in the presence of dithiothreitol, and by exposure to lysine-blocking agents such as citraconic anhydride. The fact that inhibition produced by citraconic anhydride can be fully reversed by incubation at pH 4 for several hours (data not shown) argues that the anhydride inhibition operates by acylation of an amino group critical for blocking activity.

For our long-range purposes, we wish most urgently to know the affinity of CTX for the Ca²⁺-activated K⁺ channel. Our present purification allows us to determine this accurately. According to Equations 1 and 2, the apparent Kd is measured from the proportionality between blocking activity and CTX concentration, as illustrated in Fig. 4. Under our assay conditions, we find an apparent dissociation constant of 3.5 nM, 3-5-fold lower than we had previously estimated using impure material (7).

Iodination of CTX—Since CTX contains 2 tyrosine residues, we attempted to iodinate the toxin. After trying several...
methods, we settled upon the lactoperoxidase reaction as the most efficient and reproducible in this case. In order to use radiiodinated CTX as a ligand for the Ca\(^{2+}\)-activated K\(^+\) channel, it is necessary to demonstrate that the sample is radiopure, that the radioactive sample is \(^{125}\)I-CTX, and that iodo-CTX retains the ability to bind to the channel. The first point is illustrated in Fig. 5. Lane A contains the products of the lactoperoxidase iodination reaction after removal of \(^{125}\)I-. Clearly, the sample contains impurities, most of which are not protein (data not shown). After subjecting the sample to SP-Sephadex cation exchange chromatography (as described previously for unlabeled toxin), the sample is rendered essentially radiopure (lane B).

Fig. 5 also illustrates the second point. Nonreducing gels were run with iodo-CTX and unlabeled CTX. Gels were sliced into lanes and each lane was sliced into 1-cm pieces, which were then counted in a \(\gamma\)-counter. Parallel unlabeled CTX samples were eluted overnight. Protein yields and channel-blocking activity from these eluted samples were determined. The figure shows that CTX activity and radioactivity co-migrate in this system.

We measured iodo-CTX activity by reacting under conditions known to radiolabel the toxin (stoichiometry of 1.4 \(\Gamma\) / CTX), but using nonradioactive NaI. The reaction products were assayed for their ability to block Ca\(^{2+}\)-activated K\(^+\) channels (Table IV). Table IV shows that iodo-CTX retains full channel-blocking activity.

**DISCUSSION**

This report shows that it is possible to purify charybdotoxin from the 50–60 proteins making up *L. quinquestratus* venom. This inhibitor of Ca\(^{2+}\)-activated K\(^+\) channels is a minor component of the venom, constituting approximately 0.1% of the total venom protein, by weight. From the elution profile of purified CTX on the reversed-phase column, we estimate that this preparation is at least 95% pure, and this conclusion is supported by the single amino acid identified by end-group analysis, and the appearance of a single band on SDS/urea-polyacrylamide electrophoresis gels.

The molecular mass of this material has been determined by three independent methods, which do not all agree (Table III). Mobility on SDS-urea gels under reducing conditions yields a molecular mass of about 6 kDa, whereas the amino acid analysis implies a minimum molecular mass of 9.2 kDa. For small proteins, SDS-gel electrophoresis is a notoriously unreliable method for determining molecular mass, and we tend to discount the low value obtained. This conclusion is further strengthened by the comparison of molar versus mass extinction coefficients (Table III), which implies a molecular mass of 11.5 kDa. We have previously reported that CTX activity is retained inside of 2-kDa cutoff dialysis tubing, but readily permeates 10-kDa cutoff tubing (7); this fact argues that the true molecular mass is equal to, and is not an integral multiple of, the minimum molecular mass estimated above. Accordingly, we tentatively assign a molecular mass of 10 kDa to this protein. A refinement of this value awaits the complete sequencing of the protein, which is now only partially accomplished.

Several lines of evidence demonstrate that CTX is a basic protein. Of the approximately 75 amino acid residues in this protein, 8 are lysine and 6 arginine, whereas at most 10 groups are acidic. The molecule is retained on cation exchange columns under basic conditions which elute most other venom proteins. The isoelectric point cannot be measured with existing techniques, as the CTX molecule is not focused on a gel pH gradient of 8–10.8, but the elution profile of the chromatofocusing column (Fig. 2A) argues that the isoelectric point of CTX is near 11.0. The protein contains critical disulfide bonds, since activity is lost by treatment with di-thiothreitol. These disulfides probably contribute substantially to the unusual stability of the venom protein; it is known that venom proteins from both scorpions commonly contain several intramolecular disulfide linkages (23).

The exceptionally stable CTX molecule appears to be an excellent choice of high-affinity ligand to employ in a biochemical assay for the high-conductance Ca\(^{2+}\)-activated K\(^+\) channel. Indeed, CTX is the only inhibitor displaying nanomolar affinity for this channel. We are now in a position to compare the present measurements of CTX inhibition of single channels with direct binding of \(^{125}\)I-CTX to membrane vesicles containing this channel and to commence efforts to purify the channel protein itself.

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