Purification of the tetrodotoxin-binding component associated with the voltage-sensitive sodium channel from *Electrophorus electricus* electroplax membranes

(Receptor/saxitoxin/electrical excitability/mixed micelle)

WILLIAM S. AGNEW, SIMON R. LEVINSON, JOHN S. BRABSON, AND MICHAEL A. RAFTERY

Church Laboratory of Chemical Biology, Division of Chemistry and Chemical Engineering, California Institute of Technology, 164-30, Pasadena, California 91125

*Communicated by Harden M. McConnell, March 6, 1978*

**ABSTRACT** The tetrodotoxin-binding component associated with the voltage-sensitive sodium channel from electroplax membranes of *Electrophorus electricus* has been purified. The toxin-binding site could be efficiently solubilized with Lubrol-PX, resulting in an extract of high initial specific activity. Purification was facilitated by the development of a rapid, quantitative binding assay. The binding component was stabilized during purification by the use of mixed lipid/detergent micelles of defined composition, and by the saturation of the site with tetrodotoxin.

The purification was achieved by means of a highly selective adsorption of the toxin-binding component to DEAE-Sephadex A-25, followed by desorption at high ionic strength and chromatography over Sepharose 6B. Final peak specific activities were at least 50% of the specific activity expected for a pure, undenatured toxin-binding component of 230,000 molecular weight. The purified material exhibited a sedimentation coefficient of approximately 8 S and an unusual Stokes radius of 95 Å. Purified material showed a relatively simple pattern on sodium dodecyl sulfate/polyacrylamide gel electrophoresis, being comprised of only three polypeptides.

The conductance and amplification of the electrical impulse in nerve and muscle membrane results from the action of two types of voltage-sensitive ion conductance channels (1). It is generally accepted that these are transmembrane protein structures, forming actual pores which are selectively permeable to either Na⁺ or K⁺. These channels are "gated" (opened or closed) by time- and voltage-dependent mechanisms (2–4).

The sodium channel may be quantified in disrupted and detergent-solubilized membrane preparations by the specific binding of the neurotoxins tetrodotoxin (TTX) and saxitoxin, (3, 5–7). Physiological studies with a variety of excitable tissues have shown that these toxins bind reversibly, with high affinity (Kₐ = 1–10 nM), to a site accessible only from the outside of the cell membrane, causing specific inhibition of voltage-dependent sodium currents (8). Binding studies with radiolabeled TTX and saxitoxin revealed that the toxins are mutually competitive in their binding to membrane sites (9). Generally good agreement between data from binding studies and independent physiological investigations (determination of Kₐ, channel densities, and single-channel conductances) leaves little doubt that the toxins specifically associate with the physiologically defined sodium channel, with a stoichiometry of one toxin molecule per channel (8, 10).

There is also evidence that TTX and saxitoxin bind to a molecular substructure responsible for selection of sodium ions for permeation through the channel, and that their inhibitory action can best be explained as the physical occlusion of the pore pathway through which the ions translocate (11). Thus the toxin-binding site is probably an intimate part of the channel structure. Because this site may be directly involved in ion selectivity, structural information may provide insight into mechanisms for ion dehydration and permeation. One promising approach to characterizing the TTX-binding site is solubilization and purification of the protein with which the toxin associates.

We present here a description of preparations and techniques that we have used to purify the TTX-binding component from *Electrophorus electricus* electroplax membranes.

**MATERIALS AND METHODS** TTX, obtained citrate-free from Sankyo Chemical Co., was tritiated by the Wilzbach procedure and purified from radiocontaminants by ion exchange chromatography (3). The processed toxin was 60–70% radiochemically pure and had a specific activity of 121 dpm/pmol [as determined by a bioassay procedure (12)]. Lubrol-PX (Imperial Chemical Industries) was made up in 10% wt/vol solutions, deionized with a mixed-bed resin [Bio-Rad AG 501-X8 (D)], and stored frozen.

Chromatographic media (DEAE-Sephadex A-25, Sephadex G-50, Sepharose 6B) were obtained from Sigma Chemical Co.

Standard proteins were purchased from Boehringer Mannheim (catalase), Worthington Biochemical Corporation (β-galactosidase), or Sigma Chemical Co. Pure phospholipids were obtained from P-L Biochemicals. Unfractionated total eel lipids were prepared by the method of Radin (13).

Medium-size specimens of *Electrophorus electricus* were obtained from Ardsley Aquariums (Ardsley, NY). To prepare electroplax membrane fragments, frozen electric organs were diced and homogenized in 5 vol of potassium phosphate buffer (50 mM, pH 7.7) using a Virtis 60 apparatus (40,000 rpm, 45 sec). Connective tissue clumps were removed by a gauze sieve, and the filtrate was centrifuged at 40,000 × g for 20 min. The pellet obtained was resuspended in 5–10 vol of buffer with a large Potter–Elvehjem homogenizer and recentrifuged at 100,000 × g for 30 min. The resultant pellet was the membrane preparation used in further studies.

TTX binding in detergent extracts was measured following a gel filtration procedure described by Lefkowitz et al. (14). Concentrated toxin ([3H]TTX, or [3H]TXX and unlabeled TTX) was added to an aliquot of the extract and allowed 5 min at 0°.

**Abbreviations:** TTX, tetrodotoxin; PtdCho, phosphatidylcholine.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U. S. C. §1734 solely to indicate this fact.
for binding equilibration. Of this, 0.2 ml was layered on a 1.5-ml Sephadex G-50 (fine) column (made in the barrel of a 3-ml plastic syringe) and allowed a few seconds to sink in. The column was suspended in a culture tube and placed in an MSE GT4 centrifuge (angle rotor). To recover the toxin-binding protein complexes from the column interstitial fluid, the centrifuge was accelerated at 1000 g min\(^{-1}\) to a final level of 1200 X g. The radioactivity of fluid collected in the culture tube was measured by standard scintillation counting methods. Unbound TTX (molecular weight 321) was fractionated and retained by the stationary phase of the gel.

Material was concentrated between purification steps by membrane ultracentrifugation (Amicon XM-300 membranes).

Sodium dodecyl sulfate/polyacylamide gel electrophoresis was done according to the method of Laemmli (15), using a slab gel with a linear gradient of 7.5–15% acrylamide.

Protein concentrations were determined by the fluorescamine (16) or Lowry et al. (17) methods, while lipid phosphate was measured by a modified Fiske-Subbarow technique (13, 18).

RESULTS

The sodium channels of the eel electric organ are functionally similar to those of nerve and muscle, and are TTX sensitive (19). By several standards this tissue is a good source of such channels. Organs of several hundred grams can be obtained from a single animal, and each gram wet weight contains approximately 100 pmol of TTX-binding sites (4). When prepared as described in Materials and Methods, packed membranes contained about 500 pmol of TTX-binding sites per g, representing 5–10 g of organ. Because of dense connective tissue, only a small amount of the packed wet weight represents membranes, there being generally about 10 mg of membrane phospholipid per g.

Membrane Solubilization. Best solubilization efficiencies were obtained with Lubrol-PX, a C\(_{12}\) to C\(_{14}\) polyunsaturated alkyl polyethoxylate. Optimal solubilization was found at final detergent concentrations of 1%, and at molar ratios of membrane lipid phosphate to detergent of about 1:5.

To effect solubilization, 1 vol of packed membranes was suspended in a total of 2.7 vol of 0.05 M potassium phosphate buffer, pH 7.8, and 0.3 vol of 10% (wt/vol) Lubrol-PX was quickly added. The suspension was homogenized in a motor-driven Potter-Elvejhem apparatus, and centrifuged at 100,000 X g for 1 hr. The supernatant obtained typically contained about 50% of the total TTX-binding activity in the original membranes.

TTX Binding. It has been reported that the rate constant for dissociation of \(^{3}H\)TTX from its binding site in several tissues is of the order of 0.01 sec\(^{-1}\) (7, 20, 21). However, we have found that dissociation rate of the solubilized toxin–binding site complex from eel electrotax is less than 1/20 \((k_{diss} < 0.0004\) sec\(^{-1}\)) that of the membrane-bound material (20). Because of this slow dissociation, complete recovery of the bound \(^{3}H\)TTX was possible in the gel filtration assay procedure. In Fig. 1B data are presented for a typical binding isotherm. Data from all such experiments could be fit by a simple Langmuir binding equation for a single class of sites with \(K_d = 1 - 10\) nM. The binding could be essentially eliminated by addition of an excess of unlabeled toxin, demonstrating the excellent signal-to-noise characteristics of the assay. The data in Fig. 1A illustrate displacement of \(^{3}H\)TTX by unlabeled TTX and saxitoxin at saturating levels of \(^{3}H\)TTX \((K_d/[TTX] \ll 1)\). Under these conditions, the midpoint for competition of \(^{3}H\)TTX binding by unlabeled TTX \((K_d = K_s)\) should be reached where TTX (unlabeled) = \(^{3}H\)TTX. This was observed, indicating the expected competitive displacement. Competition experiments with unlabeled saxitoxin (U.S. National Bureau of Standards) also showed complete competition, but indicated that \(K_d\) for saxitoxin \(\approx 8 K_d\) for TTX (Fig. 1A). This difference is slightly greater than was observed in previous studies of other tissues (22).

Stability as a Function of Lipid/Detergent Ratio and TTX Concentration. Upon solubilization, the TTX-binding site was markedly destabilized with respect to storage and mild purification procedures. The normal extract had a half-life of 3–5 days at 0\(^\circ\) and activity was completely lost during procedures such as gel filtration, even though such experiments often required only a few hours. Increasing the detergent concentration or raising the temperature markedly hastened the loss of binding activity.

We have found that the stability of the TTX-binding site was critically dependent on the lipid and detergent composition of the mixed micelles in which it was dissolved. Loss of binding activity at 18\(^\circ\) essentially followed a first-order decay relationship for about 90% of the decay process. The apparent first-order rate constant was a quantitatively reproducible function of the molar ratio of endogenous lipid phosphate to detergent. When this ratio fell below 1:15 the decay rate increased enormously. When detergent was present in combination with low molar ratios of unfractioinated electrotax lipids, destabilization could be counteracted and was a function only of the final lipid/detergent ratio (Fig. 2A). Of a number of commercially available lipids only phosphatidylcholine (egg or pig) and phosphatidylethanolamine (egg) were nearly as effective, per equivalent of lipid phosphate, as were unfractioinated eel lipids (Fig. 2B). To make use of these findings (to be reported elsewhere in detail), all purification steps after solubilization were performed with detergent containing 1 mol of pure phosphatidylcholine (egg) to 7 mol of Lubrol-PX. This lipid/detergent mixture is referred to hereafter as Lubrol/PtdCho.

The high free energy of TTX binding to its site \((\Delta G \approx -11\) kcal/mol\) (1 cal = 4.184 J) suggested that it might serve to further stabilize the solubilized protein. In Fig. 3 data are shown from experiments in which the decay of binding was increased by higher detergent and temperature levels and the decay.
curves at five binding site occupancies were observed. The effectiveness of TTX as a stabilizing agent is clear.

Fractionation Methods. Ion Exchange Procedures. Preliminary experiments with anion and cation exchange media (including DEAE-cellulose, DEAE-Sephadex A-25, and CM-cellulose) showed that at neutral pH (potassium phosphate buffer, pH 7.7, 0.05 M) more than 95% of detergent-extracted membrane protein did not bind. For DEAE, this was shown to result from inactivation of the ion exchangers by a compound in the extract that was nondialyzable, heat labile, protease sensitive (but not highly reactive with the Lowry protein reagents), and highly acidic. Importantly, the TTX-binding component was one of the few proteins that bound to the DEAE media, apparently competing kinetically with the unknown compound. The result of this phenomenon was a highly selective adsorption of the TTX-binding protein, which, after washing of the resin, could be efficiently removed at higher ionic strength. The unknown compound was removed with only low efficiency even under harsh conditions of pH and ionic strength. Fig. 4 shows results from an exploratory DEAE-Sephadex A-25 column. In large-scale purification we found it advantageous to use a DEAE-Sephadex batch procedure, which took less time and gave better recovery and purification of binding sites.

Sephadex 6B Chromatography. The solubilized TTX-binding protein could be chromatographed on Sepharose 6B, in 0.1 M potassium phosphate buffer, pH 7.7, containing 0.1% Lubrol/PtdCho and 20 mM [3H]TTX, with recoveries of 55–75%. The TTX-binding component behaved as an unexpectedly large protein of Stokes radius 95 Å (Fig. 5A), and elution behavior was not altered by purification or variation in detergent concentration. In the crude extract, the bulk of the solubilized proteins were eluted as lower molecular weight particles, and gel filtration was therefore adopted as a purification step.

Purification Procedure. On the basis of the chromatographic behavior described above, we established a rapid purification protocol which gave good recoveries of TTX-binding protein. This consisted of solubilization, a DEAE-Sephadex batch procedure, concentration by membrane ultrafiltration (Amicon XM-30 membrane), and Sepharose 6B chromatography, followed by concentration of peak fractions and rechromatography over a second smaller Sepharose 6B column. The results from one such purification are shown in Table 1.

In detail, the procedure used was as follows: The starting material was 24 g of packed membrane, the total yield from one medium (1-m) animal. A 1% Lubrol-PX extract was prepared as described above (Membrane Solubilization). In the batch method, the extract (45 ml) was added to 45 ml of packed DEAE-Sephadex A-25 pre-equilibrated with 0.1% Lubrol/PtdCho in 0.05 M potassium phosphate buffer, pH 7.7, and the slurry was made 0.2 M in NaCl. After 30 min the supernatant was removed and the gel was washed three times with 45 ml of 0.2 M NaCl in the 0.1% Lubrol/PtdCho/phosphate buffer. The TTX-binding component was desorbed with 0.5 M NaCl in the same buffer (45 ml) for 35 min, and the gel was washed

![Figure 2](https://example.com/fig2.png)

**FIG. 2.** Apparent first-order decay constant at 18° for loss of TTX binding at different lipid/detergent ratios. In both A and B, the symbols show the effect of adding detergent to alter the lipid/detergent ratio of a 1% Lubrol extract of membranes (highest final Lubrol concentration 4% wt/vol). Open symbols in both panels show decay constants measured for samples containing 4% Lubrol, but in which the final lipid/detergent ratio was varied by the addition of the indicated lipids dissolved in the detergent. (A) ○, Endogenous total eel lipids (unfractionated). (B) △, Phosphatidylcholine from egg; ○, phosphatidylcholine from pig.

![Figure 3](https://example.com/fig3.png)

**FIG. 3.** Stabilization of binding activity by [3H]TTX. A 1% Lubrol extract was destabilized by raising the Lubrol concentration to 4%, and decay of binding activity was measured at 18° at five concentrations of [3H]TTX. The numbers above each curve give fractional occupancy of binding sites by toxin. Curves were fit by eye.

![Figure 4](https://example.com/fig4.png)

**FIG. 4.** DEAE-Sephadex A-25 chromatography of solubilized eel membrane proteins. ●, [3H]TTX binding; ○, [3H]TTX binding in the presence of 35-fold excess of unlabeled TTX; ◄, protein determined by Lowry assay; ----, KCl concentration determined by conductance measurement. Extract (8 ml), containing 0.2 M KCl, was chromatographed on a 1.9 × 3.6 cm column of DEAE-Sephadex A-25. The eluting buffer was 0.05 M potassium phosphate at pH 7.7, 0.2 M KCl, and 0.1% Lubrol/PtdCho. Material with TTX-binding activity was eluted with a linear salt gradient from 0.2 to 0.4 M KCl. The column was pumped at 6.5 ml/hr and 1.6-ml fractions were collected. Specific activities of peak fractions ranged from 266 to 346 pmol/mg of [3H]TTX bound/mg of protein, compared with a specific activity of 24 pmol/mg for the starting extract.
FIG. 5. Stokes radius and \( s_{20,w} \). (A) The relationship of Ackers (23) was used to estimate the Stokes radius from the elution behavior of the TTX-binding component and standard proteins on a 1 \( \times \) 30 cm Sepharose 6B column. The eluting buffer was as described for Fig. 6. In order of ascending Stokes radius, standards (•) were ovalbumin, lactoperoxidase, gyceraldehyde-3-phosphate dehydrogenase, aldolase, catalase, \( \beta \)-galactosidase, and thyroglobulin; O, TTX-binding protein. (B) Fractional radial migration on sucrose density gradients as a function of \( s_{20,w} \). Standards (•) and partially purified toxin-binding protein (O) (after DEAE desorption and concentration) were run under conditions of constant sedimentation velocity (24) (linear gradient of 7–22% wt/vol sucrose, Beckman SW 41 rotor at 40,000 rpm, 18 hr). The gradients contained 0.1% Lubrol/PtdCho in 0.05 M potassium phosphate buffer, pH 7.7, and 20 nM [\( ^3H \)]TTX. In order of ascending \( s_{20,w} \), standards were lactoperoxidase, gyceraldehyde-3-phosphate dehydrogenase, catalase, and \( \beta \)-galactosidase.

one more time with 1 vol of the same buffer. Both washes (80 ml combined volume) were centrifuged to remove gel fines. The total procedure required 1.5 hr.

Sufficient [\( ^3H \)]TTX was added to the pooled washes to achieve greater than 90% occupancy of binding sites. This was concentrated to a final volume of about 4 ml and centrifuged to remove residual gel fines. Three milliliters of the concentrate was chromatographed over a 1.5 \( \times \) 65 cm Sepharose 6B column in 0.1% Lubrol/PtdCho in 0.05 M potassium phosphate buffer, pH 7.7, and 20 nM [\( ^3H \)]TTX. In order of ascending \( s_{20,w} \), standards were lactoperoxidase, gyceraldehyde-3-phosphate dehydrogenase, catalase, and \( \beta \)-galactosidase.

Table 1. Purification of the TTX-binding component

<table>
<thead>
<tr>
<th>Step</th>
<th>([ ^3H )]TTX bound, pmol</th>
<th>Protein, mg</th>
<th>Specific activity*</th>
<th>% initial binding activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% Lubrol extract</td>
<td>4290</td>
<td>311</td>
<td>13.8</td>
<td>100</td>
</tr>
<tr>
<td>DEAE-Sephadex concentrate</td>
<td>2630</td>
<td>11.5</td>
<td>229</td>
<td>61.3</td>
</tr>
<tr>
<td>Sepharose 6B column I</td>
<td></td>
<td></td>
<td></td>
<td>41.2</td>
</tr>
<tr>
<td>Total</td>
<td>1770</td>
<td></td>
<td></td>
<td>41.2</td>
</tr>
<tr>
<td>Tube 16</td>
<td>107</td>
<td>0.0694</td>
<td>1540</td>
<td>41.2</td>
</tr>
<tr>
<td>Tube 17</td>
<td>172</td>
<td>0.106</td>
<td>1620</td>
<td>41.2</td>
</tr>
<tr>
<td>Tube 18</td>
<td>225</td>
<td>0.112</td>
<td>2010</td>
<td>41.2</td>
</tr>
<tr>
<td>Tube 19</td>
<td>233</td>
<td>0.120</td>
<td>1950</td>
<td>41.2</td>
</tr>
<tr>
<td>Tube 20</td>
<td>179</td>
<td>0.130</td>
<td>1370</td>
<td>41.2</td>
</tr>
<tr>
<td>Sepharose 6B column II</td>
<td></td>
<td></td>
<td></td>
<td>26.4</td>
</tr>
<tr>
<td>Total</td>
<td>1133</td>
<td></td>
<td></td>
<td>26.4</td>
</tr>
</tbody>
</table>
| Tube 12                   | 22.2                        | 0.0128      | 1730              | 1700–2200 pmol of [\( ^3H \)]TTX bound per mg of protein. It was apparent that this step further resolved the TTX-binding protein from a small amount of the major component removed during the prior step. In assessing the extent of purification, several considerations

FIG. 6. Sepharose 6B chromatography of TTX-binding protein following the DEAE fractionation step. For both A and B, O, binding of [\( ^3H \)]TTX; binding of [\( ^3H \)]TTX, at 4°. (A) Initial large column (1.5 \( \times \) 65 cm) chromatography. Elution was carried out at 24 ml/hr (4 hr total run time), and 2.6-ml fractions were collected. Bar over main peak of binding shows fractions pooled for rechromatography. (B) Rechromatography on small column (1 \( \times \) 30 cm). Elution was performed at 8 ml/hr (2.5 hr total run time), and 0.71-ml fractions were collected. Binding competition with unlabeled TTX was not done in order to conserve material.

DISCUSSION

In electroplax membranes, the molecular weight of the TTX-binding component has been estimated by irradiation inactivation to be 230,000 (25). Values obtained by this method are generally reliable but occasionally reflect the size of an active subunit rather than an entire quaternary ensemble of protein (26). Hence the 230,000 value should be considered a lower limit for the size of the binding component. Because our detergent extracts typically had a specific activity for [\( ^3H \)]TTX binding of about 20 pmol/mg of protein, we estimated that approximately a 200-fold purification would be required to obtain binding protein of about 4000 pmol/mg of protein (representing one TTX bound per 230,000 daltons). This should be attainable by conventional methods. From the data in Table 1 it is clear that the ion exchange step contributed significantly (\( \sim \)16 fold) in this respect. Concentration by Amicon pressure dialysis gave a slight purification with generally small losses of activity.

As shown in Fig. 6A, in the first Sepharose 6B column the majority of binding activity was found in a well-defined peak. Specific activities of the peak fractions ranged from 1500 to 2000 pmol/mg of protein and the bulk of contaminating protein was well resolved from the TTX-binding activity. Rechromatography of the peak fractions showed a small increase in specific activities (1700–2200 pmol of [\( ^3H \)]TTX bound per mg of protein). It was apparent that this step further resolved the TTX-binding protein from a small amount of the major component removed during the prior step.

In assessing the extent of purification, several considerations
have been made. The specific activities of peak fractions from the second Sepharose 6B step were 45–55% of the specific activity expected on the basis of the molecular weight of 230,000 for each TTX-binding site. In addition we found (Fig. 5B) a sedimentation value of approximately 8 S for the binding protein, not exceptional for a detergent-solubilized protein of 230,000 molecular weight. The size of the molecule as determined by gel exclusion chromatography, however, indicated a large Stokes radius of approximately 95 Å. We suspect that the chromatographic behavior, while beneficial in the purification, may not be a reliable index of the molecular weight. It should be noted, however, that a larger molecular weight would imply a greater degree of purity, again assuming one binding site per molecule. It is also possible that our preparation contains partially denatured binding protein lacking the ability to associate with TTX.

A further index of the purification was a great simplification of sodium dodecyl sulfate/polyacrylamide gel electropherograms. The initial extract showed more than 50 major protein bands, whereas the fractions of highest specific activity from both Sepharose columns contained only 46,000–59,000- and approximately 300,000-molecular weight species. While we do not suggest that the subunit composition of the sodium channel is established by these preliminary observations, the great simplification in banding patterns corroborates the general conclusion that we have a substantially pure preparation.

In summary, we report here a purification of the TTX-binding protein associated with the voltage-sensitive sodium channel of the electroplax membranes from E. electricus, and we present preliminary results regarding physical properties of the system. In addition to providing a means of studying the TTX-binding site directly, the availability of a purified form of this protein should allow us to determine whether other elements of the sodium channel, such as the gating structures, have remained associated during the purification. This would also permit the study of their molecular properties.

The authors wish to thank Dr. T. Benzer and Mr. F. Stackhouse for initial development of TTX-binding assays and Mrs. Valerie Purvis for preparation of technical illustrations and manuscript typing. This work was supported by U.S. Public Health Service Grant NS 12018, by a grant from the Sloan Foundation, by National Institutes of Health Postdoctoral Fellowships to W.S.A., S.R.L., and J.S.B., and a Fellowship from the Muscular Dystrophy Association of America to W.S.A.