Molecular Morphology of the Tetrodotoxin-binding Sodium Channel Protein from *Electrophorus electricus* in Solubilized and Reconstituted Preparations

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**ABSTRACT**  The appearance of detergent-solubilized voltage-regulated sodium channel protein was recently characterized by this laboratory. Negative-staining revealed rod-shaped particles measuring 40 × 170 Å. Further studies have suggested that the actual configuration of this protein may be quite different from the rod-shaped structures. Freeze-fracture and freeze-etch images of the protein in reconstituted membranes indicated that the channel is cylindrical with a diameter of 100 Å and a minimum length of 80 Å. Experiments with two detergent systems (Lubrol-PX and sodium cholate) enabled us to explain the discrepancy between this structure and the rod-shaped particles visualized earlier. Negative staining in either detergent at low pH (4.5) produced rod-shaped structures. As the pH was increased, doughnut-shaped particles, consistent with the structure of the protein in freeze-etch, appeared in negative stain. The tendency of the protein to change shape under different pH conditions appears to be a peculiar property of this protein.

The voltage-regulated sodium channel is responsible for the early inward currents of the propagated action potentials that are characteristic of excitable cells (for reviews see 20–22). Recently, using [3H]tetrodotoxin (TTX) binding as a biochemical marker for the channel, it has been possible to purify the protein to homogeneity from the electric organ of *Electrophorus electricus* (1–3). The purest preparations of the electricplax protein have been found to contain only a large (Mr > 260,000) peptide. This is a glycopeptide, enriched in sialic acid and N-acetylhexosamines. TTX and saxitoxin binding proteins have also been isolated from mammalian muscle (4) and mammalian brain (5). Both preparations are characterized by a high molecular weight glycopeptide (Mr ~ 270,000 from brain [7] and 130–230,000 daltons from skeletal muscle [6]). In addition, however, the brain protein has smaller peptides of 37,000 and 39,000 daltons. The muscle protein has been reported to include peptides of 45,000, 37,000, and 38,000 daltons. Similar smaller peptides have not been found in the electropax preparations (3).

We recently reported the appearance of the purified solubilized TTX binding protein when negatively stained with uranyl acetate (2). The components visualized consisted of rod-shaped particles, with dimensions of approximately 40 × 170 Å, displaying a marked tendency to aggregate in ribbon-like arrays. The consistent appearance of the particles as the one identifiable structure in all samples was in keeping with the homogeneity established by compositional analysis.

The shape and dimensions of the particles, however, presented questions with respect to the protein’s orientation in the native membrane. For instance, a rod oriented with its long axis parallel to the plane of the membrane, does not suggest a structure that would form an ion channel, nor does the 40-Å thickness seem sufficient to span the low dielectric lipid region of the bilayer. An orientation with the long axis normal to the membrane might be more likely, but the 170-Å length far exceeds the bilayer thickness. By comparison, the nicotinic acetylcholine receptor is only 110 Å in total length, a length nevertheless sufficient to extend some 50 Å into the extracellular medium (8). Therefore, we undertook studies to resolve this point, using the techniques of freeze-fracture and freeze-etching on Na+ channel reconstituted into vesicles and additional negative staining procedures on solubilized protein.

**MATERIALS AND METHODS**

**Protein Purification:** Tetrodotoxin was the generous gift of Professor Y. Kishi of Harvard University. It was tritiated by the Wilzbach procedure, purified by ion exchange fractionation on Bio-Rex 70 (Bio-Rad Laboratories, Richmond, CA), and assayed for specific activity and percent purity as previ...
Levinson and co-workers (1, 9) with the modification that the G-50 Sephadex was then concentrated to 1.3 ml by ultrafiltration over an Amicon XM-300. 0.02% sodium azide for 6 h at 4°C, then against 2 liters of 5 mM sodium by either the Lubrol-PX or sodium cholate-purification protocols. Vesicles were reconstituted with material isolated in either detergent was assayed by the G-50 gel filtration system described by Levinson and co-workers (1, 9) with the modification that the G-50 Sephadex was equilibrated in buffer containing 0.1% Lubrol-PX.

The detailed protocol for the Lubrol-PX purification procedure has been described previously (1, 3). In addition, we have recently developed a method of purifying the protein with sodium cholate as the solubilizing detergent. This method achieves a larger yield of pure material (see Fig. 1) than that obtained with Lubrol-PX, and will be described in detail elsewhere (11, 12, and J. Miller and W. A. Agnew, in preparation). Briefly, membranes were solubilized by addition of Lubrol-PX to a final concentration of 1% wt/vol. This suspension was centrifuged at 100,000 g for 60 min and the supernatant adsorbed on DEAE-Sephadex A-25. The resin was washed first with 4 vol of 50 mM potassium phosphate (pH 7.4), 0.1% Lubrol-PX, 0.18 mg/ml egg phosphatidylcholine (PC), 200 mM KCl, and then with 4 vol of 50 mM potassium phosphate (pH 7.4), 1% sodium cholate, 1.6 mg/ml egg PC, and 200 mM KCl. The adsorbed TTX-binding protein was then eluted in the latter buffer made 800 mM KCl. The desorbate was concentrated by ultrafiltration over an XM-50 Amicon membrane (Amicon Corp., Danvers, MA) and applied to a column of Sepharose 68 equilibrated in “cholate buffer” (10 mg/ml cholate, 1.6 mg/ml PC, 100 mM sodium phosphate, 80 nM [3H]TTX, and 0.02% sodium azide). Fractions collected from this column were assayed for protein with a fluorescent assay and for [3H]TTX binding by the G-50 procedure (1, 3, 10). The protein isolated had specific activities of 1,800–2,000 pmol [3H]TTX binding sites/mg protein, representing a stoichiometry of ~0.6, assuming a protein molecular weight of ~300,000 g/mol (12). Analysis of polyacrylamide gel electrophoresis (PAGE) revealed that fractions with the highest specific activity consisted of the large (Mr > 260,000) peptide purified essentially to homogeneity. The fractions with highest specific activity of TTX binding were used in the reconstitution and visualization studies.

Vesicle Formation: Vesicles were reconstituted with material isolated by either the Lubrol-PX or sodium cholate-purification protocols. Vesicles were formed incorporating the cholate solubilized TTX-binding protein as in the following example: 210 wg of the purified protein in 5 ml of the cholate column buffer was dialyzed against 500 ml of 50 mM sodium phosphate (pH 7.4), 0.02% sodium azide for 6 h at 4°C, then against 2 liters of 5 mM sodium phosphate and 0.02% sodium azide for 20 h at 4°C. The opalescent product was then concentrated to 1.3 ml by ultrafiltration over an Amicon XM300 membrane (Amicon Corp.). This concentrate was frozen at ~20°C for several hours and then thawed. This freeze-thaw step produced larger vesicles that could be more easily examined.

The Lubrol-solubilized material was reconstituted into PC vesicles by a modification of the method of Weigelt and Barchi (13), making use of adsorption of the nonionic detergent to a negatively charged membrane. The adsorbed protein in Lubrol-PX (40 mg/ml protein) was supplemented with a concentrated phosphatidylcholine:Lubrol solution (36 mg/ml PC, 9% Lubrol-PX) to a final concentration of 6 mg/ml egg PC, 1.6% detergent wt/vol, and 34 µg/ml protein. To 1 ml of this solution we added 0.3 g (moist weight) of BioBeads and the slurry was stirred at 4°C for 5 h. The beads were removed by filtration and the procedure repeated for a 2-h incubation.

Freeze-fracturing and Freeze-etching: For routine freeze-fracture analysis, the vesicles formed with either the cholate or Lubrol-PX purified protein were fixed and cryoprotected before freezing. Fixation was accomplished by adding glutaraldehyde to a final concentration of 4% (vol/vol) and 3–5 min later the samples were cryoprotected by adding glycerol to a final concentration of 20% (vol/vol). A minimum of 5 min but no longer than 15 min of equilibration was allowed to elapse before all specimens were frozen. Samples destined for freeze-etching were not fixed or cryoprotected but were otherwise frozen in the same manner as those fixed and cryoprotected. This was accomplished by immersion in liquid Freon 22 cooled by liquid nitrogen. Unfixed, uncryoprotected samples were also examined following freeze-fracturing and unidirectional shadowing to determine if the fixation or cryoprotection had changed the particle size or morphology. Samples were frozen at ~120°C under a vacuum of 2 × 10⁻¹ Torr, unidirectionally shadowed with platinum evaporated from an electron gun at a 30° angle and then carbon-stabilized using a Balzers 300 freeze-etch device (Balzers, Hudson, NH) equipped with a turbomolecular pump and a quartz crystal thin film monitor (set at 180 Hz) as well as a time and heat limiting electric shutter device (14).

For freeze-etching, precise specimen stage temperature hysteresis was first carefully determined and calibrated with a thermocouple probe (Omega Engineering, Inc. Stanford, CT). Stage temperature was adjusted to ~100°C, then the specimen was fractured and etched for 5 min by placing the microtome knife (at ~196°C) directly over the specimen. Following this etching or sublimation interval, specimens were shadowed with platinum from an angle of 30° while rotating the specimen stage rapidly resulting in an omnidirectional shadowing of the etched true surface of the vesicles.

Stereoelectron micrographs of both the freeze-fracture and freeze-etch replicas were taken with a JEOL 100CX electron microscope equipped with a eucentric, side-entry goniometer stage, (+5 and −5° of tilt), operated at an accelerating voltage of 100 kV. Stereopairs were taken from selected areas of each vesicle. 100 particles identified in the flat areas in each sample group were measured with a magnifier from ×100,000 or 50,000 micrographs.

Negative Staining: For visualization by negative staining, ~10 µl of sample was applied to a carbon film of a 400-mesh copper grid, pretreated with aqueous poly-L-lysine hydrobromide (Sigma Chemical Co., St. Louis, MO) at 50 µl/ml and negatively stained with 2% aqueous uranyl acetate (UA) (Poly-sciences, Inc., Warrington, PA). Other negative stain reagents were also used at pH adjusted to 5 and 6.5 (e.g., uranyl oxalate and potassium phosphotungstate). UA however was the only stain with which we were able to achieve acceptable resolution. The usual procedure was to layer the sample on the carbon coated grid for 10 s and then rinse with either 0.1% ammonium acetate or 0.1 M Na2HPO4 buffer (pH 7.4) for 5 s, followed by staining with 1% UA at pH 4.5 or adjusted slightly upward (to approach pH 5.0), and then dried by blotting with filter paper for 1–2 min before observation. To “pre-acidify” some samples, 10 µl of 0.1 N HCl and 10 µl of 1% UA at pH 4.5 were added sequentially to a 10-µl drop of protein sample on a parafilm sheet. After 10–15 s this mixture was micropipetted onto a carbon coated grid and then blotted with filter paper as above. Occasionally a secondary staining process was used in which additional 1% UA, also at pH 4.5, was washed over the grid (held edge up) to enhance staining. These grids were allowed to air-dry. The specimens were examined with either JEOL 100B or 100CX electron microscopes, operating at 100 kV, fitted with a 100-µm objective apertures. Stereopair electron micrographs were taken at ×50,000 and 100,000 direct magnification and photographically enlarged with a point source illumination enlarger.

RESULTS

Freeze-fracturing and Freeze-etching of Reconstituted Sodium Channels in Vesicles

Vesicles containing sodium channel protein purified according to either the cholate or Lubrol purification procedures were examined. As seen in Fig. 1, the material purified by the cholate method consisted almost entirely of the large glycopeptide. Vesicles produced with protein derived by this procedure were prepared by dialysis and then enlarged by a freeze-thaw cycle. Vesicles produced with protein derived from the Lubrol procedure were formed by the somewhat more rapid BioBead method. The particles observed in these two types of reconstituted vesicles were essentially identical. Fig. 2 is an example of a vesicle from a cholate preparation. This particular sample was frozen without glutaraldehyde fixation or glyceral cryoprotection. The particles revealed by fracturing the specimen were essentially identical. Fig. 2 is an example of a vesicle from a cholate preparation. This particular sample was frozen without glutaraldehyde fixation or glyceral cryoprotection. The particles revealed by fracturing the specimen were essentially identical.
were predominantly ~100 Å in size (over 40%), ranging in diameter from 60–150 Å with a mean of 106 Å (SD = 18 Å). The vesicles formed with the Lubrol solubilized protein, fractured without fixation or cryoprotection, also exhibited particles of nearly uniform size. Over 50% of these were ~100 Å in diameter with sizes from 80–150 Å and a mean of 100 Å (SD = 13 Å). The particles of the vesicles formed from the Lubrol solubilized protein were not significantly different in size from those formed from the cholate solubilized protein. Fixation and cryoprotection had no significant effect on the particle diameter, as can be seen in the micrographs (Figs. 2 and 3) and in the quantitative analysis of particle sizes presented as a histogram (Fig. 5). After fixation and cryoprotection the average particle size was 106 Å (SD = 16 Å) with a range of diameters from 80–140 Å. The percent of particles of each size relative to total particles is also depicted in the histogram of Fig. 5. Thus, vesicles reconstituted from either Lubrol-purified or cholate-purified protein samples exhibited particles of ~100 Å in diameter. Rod-like particles of 40 × 170 Å were not visible in vesicles derived either from cholate
or Lubrol preparations. In addition, whether the material was glutaraldehyde fixed and cryoprotected with glycerol or left unfixed and uncryoprotected, particle morphology was similar.

To determine whether the particles incorporated into vesicles extended out of the bilayer, freeze-etching of vesicles was performed. Fig. 4 is from a freeze-etched and rotary-shadowed replica of vesicles reconstituted from the Lubrol solubilized protein. This specimen was not fixed or cryoprotected. The etching has proceeded to the extent that the entire vesicle shown here is exposed by etching and no freeze-fractured membrane faces were visible. The rotary shadowed surface topography (best examined in stereo) reveals numerous particulate projections, many of similar dimensions (arrows).

Negative Staining of Solubilized Sodium Channel

Our freeze-fracture and freeze-etch observations of 100-Å-diam particles that appeared to extend beyond the bilayer were not easily correlated with previous observations of highly regular 40 x 170 Å rods seen in negative stained preparations (2). Because previous observations were of the highly purified Lubrol-PX solubilized protein, we tested the generality of our earlier observations for the same macromolecule solubilized by the sodium-cholate procedure described above. Early in this second round of negative staining experiments we found it useful to raise the pH of the stains to obtain the best spreading of the cholate solubilized material. As the pH was increased, doughnut-shaped particles appeared in negative stain consistent with the structure of the protein in freeze-etch (Fig. 6, arrows). Rods were very rarely found but rather irregularly sized “doughnuts” were seen that were not unlike solubilized AChR spread on carbon filmed grids in a similar manner (15, 16). The outer diameter of the doughnuts ranged between 90 and 110 Å with most being ~100 Å.

In our previous studies on Lubrol-PX solubilized protein we used a slightly lower pH staining procedure. Therefore for comparison we studied the appearance of the cholate preparation at lower pH and the Lubrol preparation at higher pH. We found that when the cholate solubilized macromolecules were directly stained with UA at pH 4.5 on the grid, clusters of material containing doughnuts of ~100 Å were seen (Fig. 7). If the same material was incubated for 10–30 s longer in the lower pH before drying, rod-like structures, like those of the Lubrol preparations, were apparent (Fig. 8). The rod-like structures of the cholate preparation however were of non-uniform width and length dimensions, and did not align in ribbon-like clusters of the kind formed by TTX stained after Lubrol solubilization.

When Lubrol solubilized material was observed at pH >4.5 few consistently sized structures were seen. If this material was stained and dried rapidly at slightly elevated pH, (i.e., pH 5), the well defined rods previously observed with pH 4.0 staining were not apparent. Instead, unitary structures roughly similar in size to the cholate “doughnuts” could be identified (Fig. 9). Unlike the negatively stained “cholate doughnuts” these “Lubrol doughnuts” were positively stained with an electron lucent center. If preparations of the same Lubrol-PX solubilized material were first acidified and then stained (see Materials and Methods), the previously reported ribbon clusters (2) of rod-like structures consistently appeared (Fig. 10). Doughnut structures were not often observed in preparations in which the very large extended ribbon-like clusters of rods were produced. Thus, as the pH of negative staining was increased doughnut-shaped particles appeared and as the pH was lowered rod-shaped particles appeared, in either Lubrol or cholate preparations.

DISCUSSION

[3H]TTX and [3H]STX binding have been successfully employed as biochemical markers with which to follow the sodium channel during purification from eel electroplax (1), mammalian skeletal muscle (4), and mammalian brain (5). The common compositional feature of all channel preparations is a large molecular weight glycopeptide (M, ~260,000–270,000 in electroplax and brain; 120,000–230,000 in skeletal muscle). Less consistent constituents are smaller peptides.
(39,000 and 37,000 in brain [7]; 45,000, 38,000, and 37,000 in skeletal muscle [6]). Smaller peptides have not been found in the electroplax preparation. It was hoped that direct visualization of the purified samples would demonstrate whether the electroplax protein presents an appearance suggesting an ion channel. These studies could provide information concerning the size, shape, and symmetry of the molecule and might reveal characteristics relating to normal orientation in the membrane, or to the ion transporting or gating structures.

In an earlier report (2) we described the appearance of homogeneous preparations stained with UA. We observed distinctive rod-shaped particles (40 x 170 Å) that displayed a tendency to aggregate in stacks or ribbons. The presence of up to several thousand such particles in fields from every sample examined and the absence of other regular structures strongly supported their identification as the channel protein. We speculated that the particle might orient itself with the long axis normal to the membrane, although the 170-Å length seemed excessive for purposes of forming a highly conductive ion channel. Freeze-fracture and freeze-etched images of the pure protein reinserted into PC liposomes, either from cholate solubilized or Lubrol-PX preparations failed to reveal particles of 40-Å diam, which extended far into the aqueous plane, nor were horizontal rods encountered in the bilayer. Instead,

![Figures 6-10](image)

**Figures 6-10** Negatively stained solubilized sodium channel; stereo pairs of example field. Bar, 1,000 Å, x 125,000. (Insets of highly magnified particles) Bar, 250 Å, x 400,000. Fig. 6: Cholate solubilized protein stained with UA at pH 5.0 revealing clusters of reticular material and numerous doughnut-shaped structures (arrows). Fig. 7: Cholate solubilized macromolecules directly stained with UA at pH 4.5. On the grid clusters of material containing doughnuts of ~100 Å are seen. Fig. 8: If the same cholate solubilized material was allowed to sit for 10–30 s longer in the lower pH stains before drying, rod-like structures were apparent. Fig. 9: Lubrol solubilized material stained and dried rapidly at slightly elevated pH (pH 5) revealed unitary structures roughly similar in size to the cholate “doughnut”. These “Lubrol doughnuts” however were positively stained with a light center. Fig. 10: If preparations of the same Lubrol-PX solubilized material were first acidified and then stained (see Materials and Methods), the previously reported ribbon clusters of rod-like structures consistently appeared.
reproducible particles of ~100 Å diam, were found extending moderately from the true surface of the membrane.

Re-examination of negative stained Lubrol-PX preparations under the conditions employed previously again demonstrated rods described earlier. However, when stained and dried quickly at pH 4.5, rods were observed with decreasing frequency and were not observed at pH ≥ 5.0. At these pH, the material was not as distinctively uniform, but gave a vague impression of doughnut shaped structures. Cholate solubilized samples, viewed at more acidic pH contained rod-shaped particles, but the structures were not as well ordered into aggregates and appeared rope-like or segmented. At less acidic pH (>4.5), the cholate preparations appeared to take on distinctly doughnut shaped morphologies. Most of the material aggregated in clusters (not individual particles) or aggregates of 2 or 3 “doughnuts”. It is clear that the rod-shaped appearance, easily observed at more acidic pH, is not obtained at more neutral pH. Parallel pH staining studies with the Triton X-100 solubilized Torpedo acetylcholine receptor, conducted during the course of these studies and previously by us (15, 16) did not reveal comparable morphologic variations.

Several findings made here deserve special comment. The discovery of prominent, consistently sized particles extending from the true surface of the reconstituted membranes agrees with the high degree of glycosylation previously demonstrated (3, 11, 12, 17). The peptide is ~29 wt% carbohydrate and these residues almost certainly must extend out beyond the extracellular surface of the lipid bilayer. From the results here it is not certain whether the particles extend beyond both or only one surface of the membrane. Of special interest is the doughnut appearance seen under the mildest negative staining conditions. This appearance is strongly reminiscent of en-face views of the two other ion conducting channels well characterized in negative stain: the gap junction structure (19), and the nicotinic acetylcholine receptor (8). In both of these instances the proteins form structures of 85–90 Å diam with prominent, dark staining central pits, interpreted to be associated with the ionic pores. The doughnut structure of the sodium channel preparations in negative stain are somewhat irregular. This may indicate that the protein is not completely in the normal conformation adopted in the native membrane.

The precise relationship between the well defined and consistent rod structures found at low pH, to the doughnut structures observed at higher pH is at present unresolved. It is possible that removal of restraining forces provided by the membrane bilayer microenvironment would permit ~100-Å diam doughnut structure to relax into a longer, narrower (40 Å) rod by lengthening in the dimension normal to the membrane. Other alternative rearrangements may be envisioned, but none as yet are firmly supported by observation. We observed that rods in Lubrol, more prominently in cholate, often have a beaded or segmented appearance. In cholate, rods are often not perfectly linear and do not as readily aggregate in the precise side-to-side registry characteristic of Lubrol preparations.

We conclude that the rod shapes are characteristic of the protein under more acidic conditions. At more neutral pH, doughnut-like structures of larger diameter (~100 Å) are often encountered. Freeze-fracture and freeze-etched views of the particles in reconstituted membranes are more consistent with the latter particle morphology in the membrane environment. The apparent conformational flexibility of this protein contrasts to the fixed structure of the acetylcholine receptor and this may reflect greater influence on the protein conformation by the bilayer. This would be consistent with the findings that the stability of the soluble protein is acutely sensitive to the inclusion of low ratios of membrane lipids in detergents used for solubilization and fractionation (18). It also suggests that some caution should be exercised in attempts to deduce details of the protein shape and symmetry from negative staining of the solubilized protein.

Very special acknowledgement of the expert and patient technical assistance are due Thomas Deerinck on the negative staining, and Derek Leong on the freeze-fracturing. Robert L. Rosenberg and Sally A. Tomiko are thanked for preparing the Lubrol-derived reconstituted membranes. Also thanks to Dolores Taitano and Tina DeGennaro for typing the manuscript, and James Lindsey and Jean LeBeau for help with the histogram.

This research supported by National Institutes of Health grants NS17928 to W. S. Agnew and NS14718 to M. H. Ellisman; NMSS grants to W. S. Agnew and M. H. Ellisman; MDA grant to M. H. Ellisman; and a postdoctoral fellowship from the MDA to J. A. Miller.

Received for publication 15 July 1983, and in revised form 29 August 1983.

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