Principal Glycopeptide of the Tetrodotoxin/Saxitoxin Binding Protein from *Electrophorus electricus*: Isolation and Partial Chemical and Physical Characterization†

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ABSTRACT: Preparations of the tetrodotoxin (TTX) and saxitoxin binding protein isolated from the electroplax of *Electrophorus electricus* are of high specific activity (≥2000 pmol of TTX binding sites/mg of protein) and appear to be homogeneous in that they contain only the large polypeptide previously identified to make up part of the voltage-sensitive sodium channel [Agnew, W. S., Moore, A. C., Levinson, S. R., & Raftery, M. S. (1980) *Biochem. Biophys. Res. Commun. 92*, 860–866]. This permits the inference that the TTX binding site, thought to be associated with the mouth of the ion channel, is located on this peptide. This peptide presumably corresponds to the large peptide, designated the α-peptide subunit, of the synaptosomal sodium channel [Hartshorne, R. P., & Catterall, W. A. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4620–4624]. No convincing evidence for lower molecular weight peptides has yet been found for the electroplax protein. A rapid and convenient method is described for preparation of milligram quantities of the pure, sodium dodecyl sulfate ($NaDodSO_4$) denatured form of the peptide, and its amino acid and carbohydrate compositions are reported. The peptide behaved anomalously on $NaDodSO_4$–polyacrylamide gels. It was demonstrated that the molecular weight cannot be accurately quantified by this method but that the true value likely exceeds the value of 260 000 reported previously. The denatured peptide displayed an electrophoretic microheterogeneity which may be ascribed to variations in bulky carbohydrate substituents and an extremely high free mobility which is inferred to result from binding of unusually large amounts of $NaDodSO_4$.

The electrical excitation exhibited by membranes of nerve and muscle cells is a reflection of transient changes in their ion permeation selectivity. In the instance of the propagated nervous impulse, the early sodium currents responsible for the rising phase of depolarization are mediated by voltage-regulated, sodium-selective conductance channels (Armstrong, 1975; Cahalan, 1980; Keynes, 1975).

In the hope of ultimately being able to resolve the underlying molecular mechanisms for ion selectivity and transport and for voltage-dependent gating of the sodium channel, we have pursued the direct isolation of the channel protein and have begun to examine its basic chemical characteristics.

The biochemical isolation of the sodium channel protein has been predicated on the use of specific neurotoxins as ligands in binding assays. There are two classes of toxins which have been of use in binding studies. The polycyclic guanidines tetrodotoxin (TTX)[1] and saxitoxin (STX) bind to the same site to block the ion conductance mechanism, and peptides such as scorpion toxin (ScTX) from the venom of *Leiurus* and anemone toxin (ATX II) from *Anemone* interfere with gating mechanisms (Armstrong, 1975; Cahalan, 1980; Keynes, 1975). Other agents, such as the alkaloid toxins batrachotoxin and veratridine, due to their lipid solubility and comparatively low affinities, have been of minimal value in direct binding studies. It has been possible to isolate TTX and STX binding proteins from several tissue sources (Agnew et al., 1978; Barchi et al., 1980; Hartshorne & Catterall, 1981). It should be noted, however, that TTX/STX and the peptide toxins bind to different sites on the channel and affect channel function in different ways; hence, one must exercise caution before assuming that the purified TTX/STX binding protein remains associated with peptide toxin sites, or indeed any other parts of the sodium channel mechanism or structure.

We previously have reported methods for purifying the TTX and STX binding protein from *Electrophorus electricus* to high specific activities and simple peptide compositions. On the basis of the number of binding sites and the approximate molecular weight of the protein, the preparations were judged to be approximately 50% pure (Agnew et al., 1980, 1981). We further identified directly a large, ~260 000-dalton peptide, designated "band I", to be an essential component of the sodium channel molecule (Agnew et al., 1980, 1981). The purest preparations also had low and variable levels of smaller peptides of 59 000 and 42 000 daltons, which could not be excluded as contributing to the protein assembly.

Subsequently, the STX binding protein from sarcolemma was isolated to slightly lower specific activities by procedures somewhat different from those used with the electroplax (Barchi et al., 1980). Peptides of the purest material were of 64 000, 60 000, and 50 000 daltons. Shortly after this, Hartshorne & Catterall (1981) reported the purification of the STX "receptor" of the sodium channel from brain synaptosomes. The material was of similar specific activity to that from sarcolemma but contained high levels of peptides of 270 000 and 38 000 daltons. These were essentially identical in molecular weight with peptides photolabeled in situ by arylazido derivatives of ScTX, suggesting that both the STX and ScTX neurotoxins associated with a large peptide of 270 000 daltons and perhaps a smaller peptide of 38 000

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[1] Abbreviations: Bis, *N,N'*-methylenebis(acrylamide); CBB, Coomassie brilliant blue; DEAE, diethylaminoethyl; STX, saxitoxin; $NaDodSO_4$, sodium dodecyl sulfate; TTX, tetrodotoxin; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; Tris, tris(hydroxymethyl)aminomethane.

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daltons. The authors designated these the α- and β-peptide subunits of the sodium channel.

We show here that it is possible to prepare a native TTX binding molecule of high specific activity (2200 pmol of TTX binding sites/mg of protein) free of detectable levels of low molecular weight peptides. This permits the unambiguous assignment of the TTX binding site to the large peptide, presumably analogous to the α-peptide of Hartshorne & Catterall (1981). Because the methods incompletely resolved contaminants at each stage, the fractionation of the native molecule to homogeneity is not highly efficient. However, we describe a convenient method for preparation of milligram quantities of the pure peptide in the $NaDodSO_4$-denatured form. It is shown to be heavily glycosylated. We report its amino acid and carbohydrate composition and characterize its unusual behavior during electrophoresis on polyacrylamide gels. We conclude that, although its molecular weight cannot be accurately determined by this procedure, the true value is likely to exceed the value of 260 000 reported previously. We also show that the peptide displays an apparent electrophoretic microheterogeneity but that it is not likely to be contaminated by other peptides nor to be an aggregate of smaller peptides. We infer from data on electrophoretic mobility that it binds unusually large amounts of $NaDodSO_4$.

Materials and Methods

TTX (citrate free) was the kind gift of Professor Y. Kishi, Harvard University. It was tritiated by the Wilzbach procedure and purified by ion-exchange chromatography as described by Benzer & Raftery (1972). The toxin was approximately 60% pure as determined by frog sciatic nerve bioassay (Levinson, 1975). The toxin specific activity was 58.6 μCi/μmol. [^{3}H]TTX binding to detergent extracts was measured by the G-50 assay of Levinson et al. (1979).

Protein assays were by the fluorescamine procedure, with bovine serum albumin as the standard (Udenfried et al., 1972).

Lubrol-PX (Sigma Chemical Co.) was made up in 10% (w/v) solutions, deionized with the mixed-bed resin Bio-Rad AG 501-X8(D), and stored frozen. Pure egg phosphatidylcholine was obtained from Sigma Chemical Co.

Electric eels (0.8–1.2 m) were obtained from World Wide Scientific Products. Animals were killed by hypothermia and the organs removed, trimmed of connective tissue, and frozen at −80 °C. For the preparation of membranes, the organ was partially thawed, diced into 1–2-cm^3 cubes, added to 4 volumes of buffer (50 mM sodium phosphate, pH 6.8, 5 mM EDTA, and 0.1 mM PMSF), and homogenized in a Tekmar SDT-45 homogenizer for 30 s at full speed. Homogenates were strained through two layers of cheesecloth and centrifuged for 20 min at 30000g in the JA-14 rotor of the Beckman J2-21 centrifuge. Supernatants were again discarded, and the pellets were resuspended in approximately 5 volumes of buffer and packed by centrifugation at 48000g in the JA-20 rotor. Supernatants were discarded and the weighed pellets resuspended with 1 mL of buffer/g of membranes. Suspensions were stored in aliquots at −80 °C. Yields of such crude packed membranes were usually about 25% of the original tissue weight.

The purification of the native TTX binding protein illustrated in Figure 1 and Table I was as follows: 72 mL of membranes was thawed and homogenized with 8 mL of 10% Lubrol-PX in a Potter Elvejhem homogenizer and centrifuged at 100000g in a Beckman 40.1 rotor. The supernatant was removed, and the 55 mL of extract obtained was stirred into an equal volume of DEAE-Sephadex A-25 ion-exchange resin which had been previously equilibrated with a buffer of 0.05 M sodium phosphate, pH 6.8, containing 0.1% of a 7 to 1 molar ratio of (w/v) Lubrol-PX to phosphatidylcholine. After incubation for 30 min, the resin was washed with 5 volumes of buffer containing 0.2 M NaCl and then mixed with 55 mL of 0.6 M KCl for 30 min. The supernatant was removed, and the resin was washed with 25 mL of 0.4 M KCl. The latter two washes were pooled and concentrated over an XM-300 membrane in an Amicon pressure dialyzer to 2.3 mL. The concentrate was assayed for [^{3}H]TTX binding and protein; samples were saved for gels. To the remaining 2.15 mL was added 0.2 mL of 5 μM [^{3}H]TTX. The sample was delivered to a 1.6 × 65 cm Sepharose 6B column, freshly equilibrated with 50 nM [^{3}H]TTX, 0.1 M sodium phosphate, pH 6.8, and 0.1% (w/v) Lubrol-PX/phosphatidylcholine (7:1 molar ratio). The column was eluted at 8 mL/h into 3.6-mL fractions. Fractions were assayed for TTX binding protein and analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis. For the preparation of a small amount of the most highly purified material, the leading edge fractions from this column (e.g., fractions 20–22) were pooled, concentrated, and eluted through a 1 × 48 cm Sepharose 6B column in the same manner.

For isolation of the $NaDodSO_4$-denatured peptide by Sepharose 4B chromatography, a jacketed column of 1.6 × 90 cm was run at 18 °C, equilibrated with 0.1% $NaDodSO_4$ and 1.0% 2-mercaptoethanol in 50 mM sodium phosphate buffer, pH 7.5. For the experiment illustrated in Figure 3 and Table II, a purification essentially identical with that described above was performed, starting with 108 mL of membrane suspension, representing approximately 225 g of organ (half of the tissue from one large eel). Fractions 21–24 from the Sepharose 6B column were pooled and concentrated to 0.9 mL, and an equal volume of sample buffer (10% glycerol, 5% 2-mercaptoethanol, 3% $NaDodSO_4$, and 0.0625 M Tris-HCl, pH 6.8) was added. Fifty milliliters of water was brought to a boil and removed from the heat, and the sample was heated in this for 2 min. More vigorous boiling conditions resulted in aggregation of the 95 000-dalton contaminant and loss of resolution during the subsequent chromatography. The sample was then delivered to the column which was eluted in 14 fractions of 6 mL, followed by 66 fractions of 2 mL. Each fraction was assayed for protein, and selected fractions were analyzed for peptide composition on standard 5% $NaDodSO_4$–polyacrylamide gels.

Standard gels were by the method of Laemmli (1970), containing 5% acrylamide and 0.13% *N,N'*-methylenebis-(acrylamide). Ferguson gels (Ferguson & Wallace, 1961) were prepared also be the method of Laemmli, at the indicated acrylamide concentrations, with the same ratio of Bis. The periodic acid–Schiff procedure of Gordon (1975) was used to stain for carbohydrate.

The two-directional $NaDodSO_4$ × $NaDodSO_4$ gels were run as follows. The first direction was a tube gel of 5% acrylamide and 0.13% Bis according to Laemmli (1970). TTX binding protein of moderate purity was electrophoresed until the dye front reached the end of the tube. The gel was then removed and soaked in 50 mL of upper Tris buffer (0–125 M Tris-HCl, pH 6.8, and 0.1% $NaDodSO_4$) for 2 h. The second dimension was a 1.5-mm-thick slab gel consisting of a 2-cm bottom plug of 10% acrylamide and 0.8% Bis in lower Tris buffer (0.375 M Tris, pH 8.8, and 0.1% $NaDodSO_4$). This gel was polymerized and then overlayed with a 10-cm separating gel of 4.25% acrylamide and 0.11% Bis in lower Tris buffer. This was next overlayed with a 3-cm stacking phase of 3% acrylamide and 0.08% Bis in upper Tris buffer. The equilibrated tube gel was placed horizontally on top of this slab and sealed in place with molten 1% agarose (Seakem LE; Marine Colloids, Rockland,

ME) in upper Tris buffer. Two wells were cut into the solidified agarose at one side of the gel in order to run a sample of the TTX binding protein and standards for comparison. The second dimension was electrophoresed at 40 V for 17 h, after which it was fixed and stained in 0.1% CBB in 10% acetic acid, 20% methanol, and 70% water overnight. It was destained in the same solution without CBB.

Carbohydrate analyses were performed by gas chromatography. Peak fractions from the $NaDodSO_4$-equilibrated Sepharose 4B column were dialyzed extensively in water containing 0.02% NaN_3. Aliquots of the dialyzed sample containing about 60 μg of protein were mixed with 2 μg of inositol (as an internal standard) and lyophilized. The dry residue was dissolved in 0.5 M HCl in anhydrous methanol, sealed in a Pyrex tube, and then hydrolyzed at 110 °C for 17 h. The solution was next dried under a stream of nitrogen and derivatized according to the procedure of Reinhold (1972). Briefly, free amino groups were acetylated with acetic anhydride, and trimethylsilyl derivatives of the sugars were formed by addition of hexamethyldisilazane and trimethylchlorosilane. Derivatized samples were then injected onto an OV-17 column mounted in a Perkin-Elmer 900 gas chromatograph. The column was programmed with a temperature gradient of 120–250 °C at 8 °C/min, and eluted compounds were monitored with a flame ionization detector. Compounds were identified by comparing retention times with those of identically treated sugar standards. The mass of each sugar was determined by comparing the area under the peak with that for a known amount of the standards. Control experiments with α-glycoprotein verified the accuracy of the workup protocol.

Amino acid determinations were performed by Dr. Werner Hirs, as follows. The $NaDodSO_4$-denatured major peptide was purified as described above and then dialyzed exhaustively against water (three changes of 500 volumes over 24 h). The sample was lyophilized, and the material was acid hydrolyzed and analyzed for amino acid composition by the methods of Moore & Stein (1963). Values reported are for a total of six determinations on two separately purified peptide samples, except for tryptophan and cysteine which were determined individually. Tryptophan was analyzed by the *p*-toluenesulfonic acid hydrolysis procedure of Liu & Chang (1971), and cysteine was determined with the performic acid oxidation method of Moore (1963).

Results

Fractionation of the Native TTX Binding Proteins. Table I shows the results from a typical purification of the native TTX binding protein. The purification protocol described previously (Agnew et al., 1978) was carried through one elution on Sepharose 6B. The data presented include the specific activities of each fraction from the column. The highest specific activities in this instance were over 2000 pmol of [^{3}H]TTX bound per mg of protein as measured by the fluorescamine assay. This corresponds to approximately 500 000 daltons per toxin binding site.

Figure 1 shows that, based on both the elution pattern and the peptide profiles, resolution of the TTX binding protein from the major contaminants was partial, with only the leading fractions being nearly homogeneous (cf. densitometer scans of fractions 20–22, Figure 2A). If the fractions of highest specific activity and simplest peptide composition, namely, those at the leading edge of the TTX binding peak, are refractionated on a second smaller column, it is possible to obtain material of high specific activity (Table I, bottom) which is essentially homogeneous, free of the 95 000-dalton peptide contaminant and other low molecular weight species (Figure 2B). (It is thought that the former may be the α subunit of the abundant Na/K-ATPase.)

Because such preparations are free of other peptides, it is now possible to conclude specifically that the TTX binding site is located in the large molecular weight (260 000) peptide.

Table I: Purification of Native TTX Binding Protein[a]

	[^{3}H]-TTX bound (pmol)	[protein] (mg)	sp act. (pmol/mg of protein)	yield, % initial extract
extract	5885	352	16.8	100
DEAE batch	3519			60
Amicon concentrate	3004	6.1	495	51
Sepharose 6B				
fraction: 18	22	0.042	516	
19	90	0.068	1331	
20	254	0.119	2138	
21	428	0.208	2061	
22	543	0.289	1884	
23	496	0.374	1329	
24	333	0.481	690	
25	198	0.585	339	
26	84	0.589	143	
total	2448			42
repurification on Sepharose 6B (typical values)				
applied to column	564	0.279	2021	100
recovered (as electrophoretically homogeneous)	133	0.061	2171	24

[a] Values given for the protein were determined by the fluorescamine assay relative to bovine serum albumin As mentioned in the text, these values and those for specific activity may be corrected for the protein mass by using the factor obtained from amino acid analysis (see text).

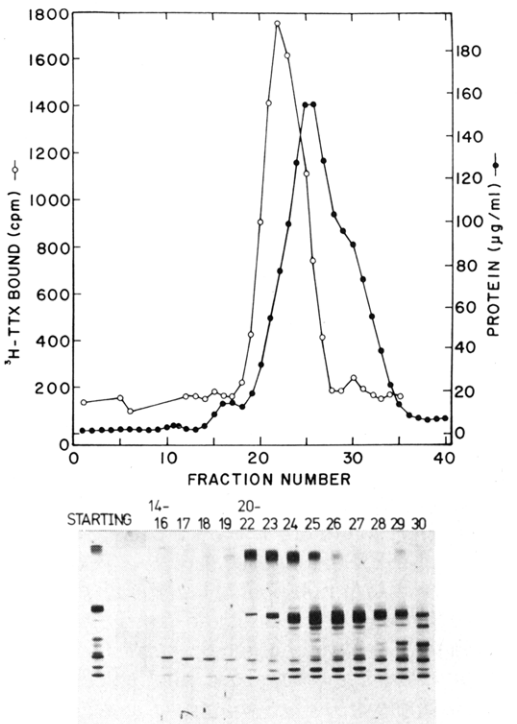

FIGURE 1: Purification of native tetrodotoxin binding protein by chromatography on Sepharose 6B. The top of the figure shows the profile of protein (closed circles) vs. [^{3}H]TTX binding activity (open circles). The bottom of the figure shows $NaDodSO_4$–polyacrylamide gel electrophoresis analysis of the polypeptide composition of column fractions. Equal volumes of each fraction were applied to the gels, except fractions 20–22 which were combined before gel analysis in order to conserve material for later studies.

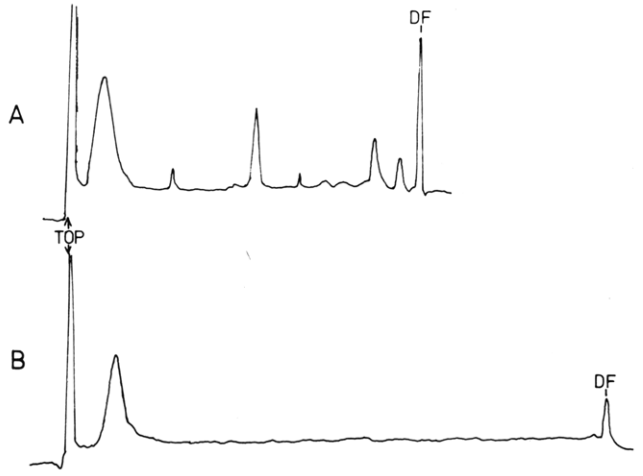

FIGURE 2: (A) Densitometer scans of gel from fractions 20–22 in Figure 1. (B) Densitometer scan of native TTX binding protein from the leading edge fraction repurified on a smaller Sepharose 6B column. Top = top of gel; DF = position of tracking dye (dye front). Specific activity of this material was 2200 pmol of TTX binding sites per mg of protein.

Table II: Purification of the Major Polypeptide of TTX Binding Protein[a]

	TTX binding activity (pmol)	[protein] (mg)	% yield
extract	8965		100
DEAE batch	4603		51
Sepharose 6B	2228[b]		25
$NaDodSO_4$-Sepharose 4B pool I		0.376	16[c]

[a] Protein concentration based on fluorescamine fluorescence relative to bovine serum albumin. The yield from the Sepharose 6B step is lower than that previously reported because only the peak fractions from this procedure were subsequently used in the final $NaDodSO_4$-Sepharose 4B step.

We then desired to establish a procedure whereby this peptide could be prepared in milligram quantities, free of lower molecular weight contaminants, for physical and compositional analysis.

$NaDodSO_4$ Gel Chromatography. When material pooled from all of the TTX binding fractions from the first Sepharose 6B column was denatured in $NaDodSO_4$, the peptides were subsequently well resolved by chromatography on a Sepharose 4B column. Figure 3 illustrates the profile of such a column, run as described under Materials and Methods. Gels run on fractions throughout the column show that the first peak (I) was entirely composed of the large peptide, peak II of the 95 000-dalton peptide, while peaks III and IV contained the smaller species. Peak V was due to Tris buffer present in the denaturation buffer which reacted with fluorescamine.

This procedure enables the preparation of 0.35–0.50 mg of totally pure peptide in one medium-scale purification, representing half the tissue from a single animal. Table II presents the yield of material from the purification illustrated in Figure 3.

Electrophoretic Behavior of the Purified Peptide. The $NaDodSO_4$ column purified peptide was examined carefully under different denaturing polyacrylamide gel electrophoresis systems to assess its homogeneity and approximate molecular weight. Several unusual properties were observed.

(*a*) *Conditions for Denaturation.* The peptide behaved as a diffuse band, based on $NaDodSO_4$ gels. To be sure this was not an artifact of incomplete denaturation, or due to the

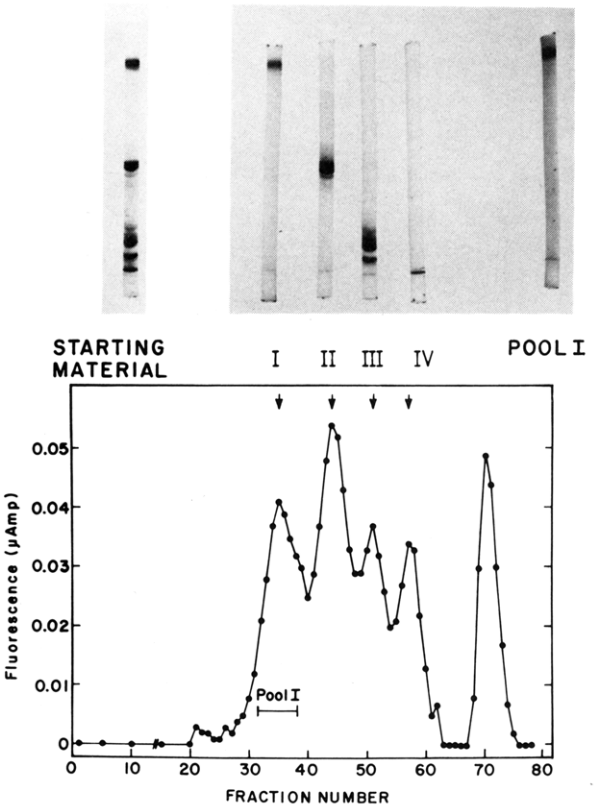

FIGURE 3: Purification of the major polypeptide of the TTX binding protein by $NaDodSO_4$ chromatography on Sepharose 4B. Bottom, protein profile, as determined by fluorescamine fluorescence. Top, $NaDodSO_4$–polyacrylamide gel electrophoresis analysis of eluted protein peaks. The arrows beneath each gel point to the fraction used for analysis, except for the POOL I gel, which was run by using the pooled fractions as shown in the profile.

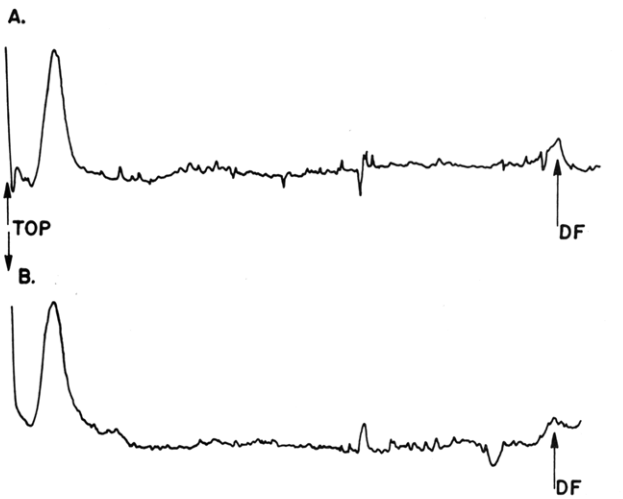

FIGURE 4: $NaDodSO_4$–polyacrylamide gel electrophoresis of the purified polypeptide (pool 1) run by using (A) the standard system of Laemmli (as in Figure 3) or (B) the $NaDodSO_4$–urea procedure of Swank & Munkries (1971). Note that the band characteristics seen in these densitometry scans are essentially the same.

presence of contaminating peptides, we examined the behavior in several gel systems. On standard 5 or 5.5% Laemmli gels, the peptide ran as a single component with an apparent molecular weight of approximately 260 000–300 000. Boiling for 5 min in 3% $NaDodSO_4$ and 5% 2-mercaptoethanol did not result in dissociation to lower molecular weight components (Figure 4A). Reduction with 2-mercaptoethanol or dithiothreitol, followed by carboxyamidation with iodoacetamide, did not change the gel pattern (data not shown). When

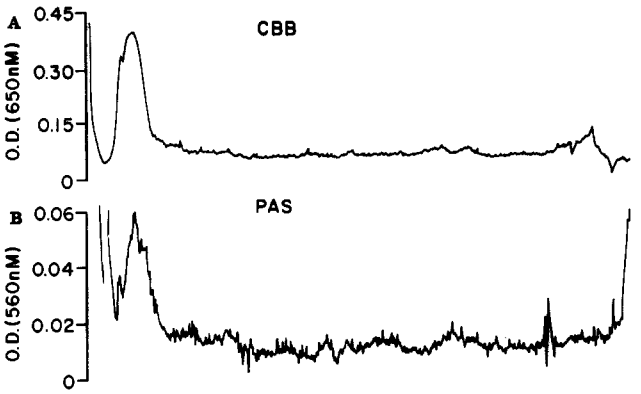

FIGURE 5: $NaDodSO_4$–polyacrylamide gel (5%) stained for carbohydrate by the periodic acid–Schiff (PAS) method compared to a companion gel stained with Coomassie brilliant blue (CBB).

electrophoresed in gels containing 6 M urea and 0.1% $NaDodSO_4$ (Swank & Munkries, 1971), the behavior of the peptide was unaltered (Figure 4B; the apparent small peak in this figure was caused by a defect in the gel surface). Thus, after various rigorous methods of denaturation, it consistently behaved as a single large molecular weight species.

(*b*) *Carbohydrate Staining.* The native TTX binding protein from eel (Moore et al., 1982), sarcolemma (Barchi et al., 1980), and synaptosomes (Hartshorne & Catterall, 1981) is absorbed by lectin affinity columns and may be eluted with the appropriate glycosides. It was thus expected that the large peptide should show evidence of glycosylation. Gels stained for carbohydrate by the periodic acid–Schiff procedure exhibited a large positive reaction for carbohydrate coinciding precisely with the Coomassie brilliant blue (CBB) protein stain of a companion gel (Figure 5A,B).

(*c*) *Molecular Weight Estimation.* Estimates of the peptide molecular weight have been provided solely by comparison to other standards run on denaturing gels. Its apparent size is nearly as great as that of the entire nicotinic acetylcholine receptor (Karlin, 1980) and approximately that expected for the entire TTX binding component (Levinson & Ellory, 1973). Thus, it was important to assess whether $NaDodSO_4$ gel electrophoresis is a reliable method of molecular weight estimation.

The basis for $NaDodSO_4$–polyacrylamide gel electrophoresis molecular weight determinations lies in the observation that most polypeptides bind a large but constant amount of negatively charged $NaDodSO_4$ per unit mass [i.e., 1.4 g of detergent/g of protein (Reynolds & Tanford, 1970; Nozaki et al., 1976)]. This effectively swamps out the intrinsic charge of a polypeptide and provides the electrophoretic driving force. Further, most sulfhydryl-reduced $NaDodSO_4$-denatured proteins form a homologous series of extended rod-shaped structures, larger peptides forming longer rods with higher frictional coefficients for transport. The ratio of driving force to frictional coefficient determines the free solution mobility (Tanford, 1961) and is about constant for most peptides, regardless of size. When electrophoresed on polyacrylamide gels, steric retardation by the gel matrix slows large peptides more than small peptides. Thus, the regular relation between mobility and molecular weight permits size estimates of an unknown. Some peptides, however, migrates anomalously. This can be due to the failure to unfold into an extended rod in $NaDodSO_4$ and/or the binding of less $NaDodSO_4$ than expected. The latter appears to occur often with glycoproteins [see Gordon (1975)]. Therefore, molecular weight estimates made in comparison to well-behaved standards are invalid for these anomalous peptides.

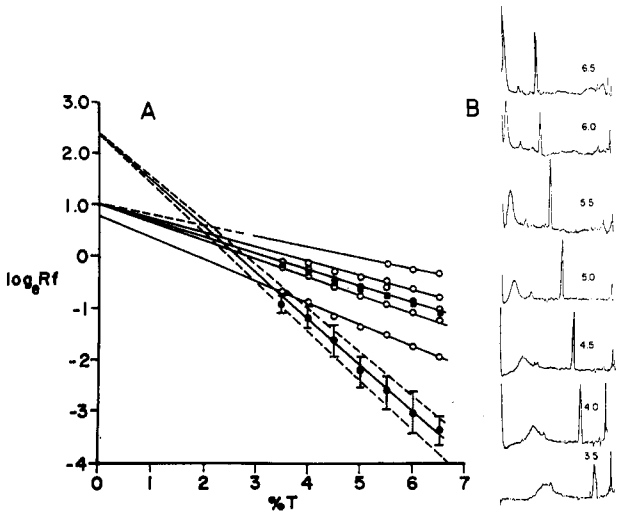

FIGURE 6: Ferguson analysis of the electrophoretic behavior of the large polypeptide compared with that of standard proteins. (A) Plot of the natural logarithm of the relative electrophoretic mobility (R_f) vs. the acrylamide concentration used to cast the gel (T). Standards (open symbols) from top to bottom were ovalbumin, bovine serum albumin, phosphorylase *b*, β-galactosidase, and myosin. The migration of the large polypeptide is shown by the closed circles, while that of the 95 000-dalton contaminant present is shown by the closed squares. The error bars for the large polypeptide represent the migration of the leading and trailing edges of the band (positions at half-height) as described in the text. (B) Densitometer scans of the polyacrylamide gels used for Ferguson analysis shown in (A). The numbers given for each scan represent the acrylamide concentration used. These scans are included to show both the wide bandwidth of the peptide, the fact that the band does not resolve into distinguishable components, and the fact that the large peptide increases its mobility *relative* to other peptides as the gel percentage is reduced.

Table III: Apparent Molecular Weight of the Large Polypeptide as a Function of Acrylamide Concentration Used in the $NaDodSO_4$–Polyacrylamide Gel Electrophoresis Estimate[a]

% acrylamide	molecular mass of TTX binding protein (kdalton)
3.5	230 (195-266)
4.0	272 (236-312)
4.5	274 (230-310)
5.0	292 (263-317)
5.5	258 (243-274)
6.0	245 (227-254)
6.5	229 (222-235)

[a] Values given for the range refer to apparent molecular weights at the half-heights of the leading and trailing edges of the resultant broad peak.

Ferguson & Wallace (1961) have described a convenient way to assess the electrophoretic behavior of an unknown polypeptide. In this approach, the unknown and standard proteins are run on gels of different acrylamide concentrations, but keeping the ratio of acrylamide to bis(acrylamide) constant. In this way, the porosity of the gel is varied, and the log of peptide mobility is linearly related to the percent acrylamide used to form the gel (cf. Figure 6A). Extrapolation of the mobility to that in 0% acrylamide gives an estimate of the free solution mobility of the unknown, compared to those of the standards. The slopes of the lines in semilog plots are considered to be related to the size (steric retardation) of the denatured protein.

Ferguson analysis of the sodium channel peptide is shown in Figure 6 and Table III. The experimental sample analyzed was only partially purified. This permits an illustration of the unusual behavior of the large glycopeptide in comparison with the more conventional behavior of other contaminating pep-

tides derived from the electroplax membranes. One of the standards, myosin, behaves slightly anomalously, having a free mobility about 0.88 that of the other standards. This has been reported elsewhere (Pitt-Rivers & Impiombato, 1968) and is not large enough to greatly affect estimates of its molecular weight. Data for the standard proteins are presented as open symbols in Figure 6.

When the relative mobility of the 95 000-dalton contaminant was plotted (Figure 6A, closed squares), it conformed with the behavior of the standards. The extrapolated free mobility was the same as that of the standards, and its mobility in relation to the standards was not altered by increasing concentrations of acrylamide. By contrast, the large peptide behaved far differently from any of the other species. The extrapolated free mobility was 3.6 times greater than that of the standards. Therefore, its mobility relative to the standards, and thus its estimated molecular weight, varied as a function of the acrylamide concentration (see Table III).

As readily seen in Figure 6A, the mobility of the large peptide increased much more quickly than that of the standards as acrylamide concentration was reduced. Note that with large pore sizes (lower T, Figure 6B), the large peptide actually overtook a minor component which ran ahead of it at smaller pore sizes (higher T). Thus, it is clear that an accurate molecular weight cannot be determined by this method.

Although we cannot accurately quantitate the molecular weight, inspection of the electrophoretic parameters suggests that the true value may be *larger* than the 260 000 daltons reported earlier (Agnew et al., 1980, 1981). Note that the peptide's *free mobility* is greater than that for the standard peptides. This might result from the binding of greater amounts of $NaDodSO_4$ (higher electrophoretic driving force) or from a smaller frictional coefficient perhaps caused by a failure of the peptide to unfold. The latter is unlikely because the *slope* of the Ferguson plot for the peptide (reflecting steric retardation) is much greater than that for any of the peptides, even the 210 000-dalton myosin. These observations suggest a structure both highly negatively charged and much larger than the largest standard. These anomalies almost certainly cannot be ascribed to effects of carbohydrate residues linked to the peptide (see Discusion).

(*d*) *Diffusely Staining Band.* In addition to its unusual mobility, the large peptide ran as an increasingly diffuse band in lower percentages of acrylamide. In Figure 6B are shown densitometer scans of all several experimental gels. As gel percentages decreased, each contaminant peptide predictably migrated further toward the dye front, and each band broadened only slightly. For instance, the 95 000-dalton peptide displayed a bandwidth of 0.02 of the gel length in both 3.5 and 6.5% acrylamide. By contrast, however, the large peptide occupied 0.02 of the gel at 6.5% acrylamide, but more than 0.17 of the gel at 3.5%. The band, however, did not resolve into multiple peaks. Either of two explanations might account for this phenomenon. First, the diffuse band could be caused by homogeneous components continuously undergoing interconversions during electrophoresis. For example, rapid intra- or intermolecular interactions might cause fluctuating shape changes in the population. Alternatively, the band broadness might result from a larger number of truly distinct and stable species whose mobilities overlap, thus escaping resolution as individual bands. Such behavior has been shown to be the result of variable degrees of glycosylation in other systems (Horowitz, 1977). We do not, however, consider this electrophoretic microheterogeneity to be a manifestation of contaminating peptides which run with the peptide of interest. Figure 6A shows that both the leading and the trailing edges of the peak display the same unusually high free mobility as the peak itself. This makes it highly unlikely that unrelated proteins are contaminating the band to any significant extent.

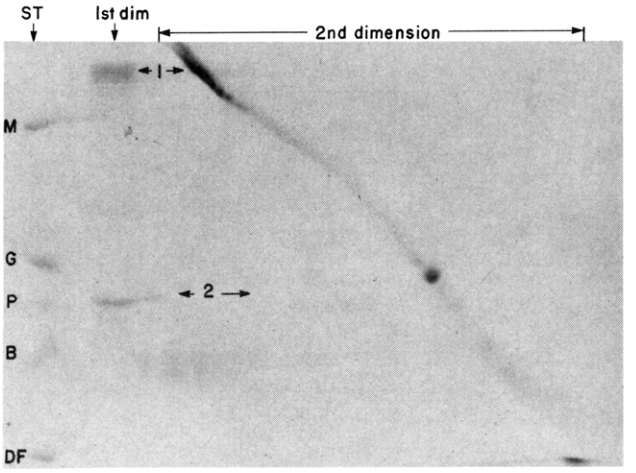

FIGURE 7: $NaDodSO_4 \times NaDodSO_4$ two-dimensional gel (5% × 4.25%) which demonstrates that the large peptide is electrophoretically heterogeneous. Samples were run as follows: ST = standards, M = myosin, G = β-galactosidase, P = phosphorylase *b*, B = bovine serum albumin, DF = dye front, and 1st dim = partially purified TTX binding protein used for two-dimensional analysis. The length of the gel for the second dimension is also illustrated. Numbers with arrows show major bands present in partially purified materials; i.e., band 1 is the large polypeptide, and band 2 is the 95 000-dalton contaminant peptide.

To distinguish between the two proposed explanations, we ran a sample of the purified peptide on two-directional $NaDodSO_4 \times NaDodSO_4$ gels. If the peptide were electrophoretically homogeneous, but undergoing rapid association or changes of shape, the final spot should be broad. If, however, the band displayed a stable electrophoretic microheterogeneity, those components migrating most rapidly in the first direction should also move fastest in the second, while those which migrated slowly in the first direction should be slow in the second. The resulting spot would be a rather sharp diagonal line. As shown in Figure 7, the result was the latter, thus indicating the peptide must be electrophoretically heterogeneous.

This result prompted more careful electrophoretic analysis of material eluting from the leading and trailing edges of peak I from the Sepharose 4B column, which separates only on the basis of size variations. Slight, reproducible differences were found in the mobility and in the bandwidth. Leading edge fractions from peak I showed an R_f of 0.072 ± 0.003 compared to 0.098 ± 0.004 for the trailing fractions on 4.25% gels. Their respective bandwidths were 0.052 ± 0.004 and 0.088 ± 0.004 of the distance migrated by the dye fronts. These results appear to support the explanation that heterogeneity of the polypeptide band in $NaDodSO_4$–polyacrylamide gel electrophoresis is caused by stable size differences.

Precedents suggested that it might be possible to ascribe this peak width phenomenon, or "electrophoretic microheterogeneity", to the influence of carbohydrate moieties (Horowitz, 1977). We next carried out the complete analysis of the amino acid and carbohydrate composition of the peptide.

Amino Acid Composition. Samples of the peptide purified by $NaDodSO_4$ chromatography were analyzed for total amino acid composition as described under Materials and Methods. Data from these analyses are given in Table IV. There was not such a preponderance of hydrophobic amino acids as to suggest unusual interactions with detergents, nor acidic or polar

Table IV: Amino Acid Composition and Hydrophobicity Parameters of TTX Binding Protein and of Related Membrane and Peripheral Proteins of the Electroplax of *Electrophorus*[a]

	TTX binding component	eel ACHR	eel ATPase (large subunit)	eel ACHease
Asx	10.1	11.4	10.6	9.7
Thr	5.0	5.6	6.0	4.4
Ser	7.1	6.2	7.6	6.9
Glx	10.8	10.2	9.8	9.5
Pro	4.7	5.7	5.2	8.4
Gly	6.5	5.9	8.3	8.0
Ala	6.0	5.8	7.6	5.7
Cys	2.0	2.0	ND	1.1
Val	5.3	8.6	7.2	7.2
Met	3.4	2.0	2.6	2.9
Ile	5.5	6.4	6.4	3.8
Leu	9.3	10.5	10.7	9.1
Tyr	2.8	4.0	2.0	3.8
Phe	5.9	5.7	4.5	5.3
His	2.8	2.2	1.6	2.3
Trp	3.5	ND	ND	2.1
Lys	5.0	4.6	5.7	4.4
Arg	4.3	6.2	5.0	5.5
$H\phi_{av}$[b]	1073	1177	(1248)[c]	957
z[b]	0.29	0.40	(0.42)[c]	0.24

[a] Abbreviations: ACHR, acetylcholine receptor; ACHease, acetylcholinesterase; ND, not determined. [b] Hydrophobicity parameters (see text). [c] Values given are for native eel ATPase (large and small subunits).

species which might underlie the unusual electrophoretic behavior. For comparison, amino acid compositions reported in the literature for three other proteins from *Electrophorus* electroplax are tabulated. The acetylcholine receptor and the α subunit of the Na/K-ATPase are intrinsic membrane proteins, and acetylcholinesterase is a peripheral protein (Barrantes, 1975).

The presence of norleucine as an internal standard allowed calculation of the true amounts of protein present in the samples subjected to amino acid analysis. It was found that the fluorescamine assay standardized with bovine serum albumin (BSA) resulted in protein values 85.5% of the true value. This correction factor has been applied to the carbohydrate analysis which follows.

Carbohydrate Composition. Material purified by NaDodSO$_4$ gel chromatography was analyzed for total carbohydrate composition as described under Materials and Methods. Determinations from two different samples are reported in Table V. The sugars detected were mannose, fucose, galactose, *N*-acetylhexosamine, and sialic acid. The latter two species were most abundant. Total carbohydrate composition was 29.5 ± 2.4% by weight. Thus, as expected from the positive reaction on gels stained with the periodic acid–Schiff reagent, the peptide has a significant carbohydrate content. Because carbohydrate moieties can form bulky substituents, they contribute significantly to steric retardation during NaDodSO$_4$–polyacrylamide gel electrophoresis. Further, variabilities in the considerable carbohydrate composition (whether due to partial degradation or to intrinsic variations in the degree of glycosylation) could underly the peak width phenomenon (electrophoretic microheterogeneity) seen on the gels.

Discussion

We have demonstrated the isolation of the native TTX binding protein in a form apparently devoid of low molecular weight peptides. We infer that the TTX binding site, presumably in close association with the ion channel (Armstrong,

Table V: Carbohydrate Composition of the TTX Binding Protein Glycopeptide[a]

	% total weight	% of carbohydrate
fucose	0.5 ± 0.2	1.5 ± 0.5
mannose	2.4 ± 0.5	8.3 ± 1.1
galactose	1.5 ± 0.3	5.2 ± 0.6
N-acetylhexosamine	13.3 ± 0.4	45.3 ± 2.6
sialic acid	11.8 ± 1.2	39.7 ± 1.6
total	29.5 ± 2.4	

[a] Protein concentration is based on quantitative amino acid analysis (see text).

1975; Cahalan, 1980; Keynes, 1975), is on this peptide. The highest specific activity fractions are of approximately 2200 pmol of TTX binding sites/mg of protein, corresponding to about 55% of the theoretical specific activity if the protein were 250 000 daltons. The peptide composition, however, suggests that the best preparations are essentially homogeneous and that some of the molecules present may be denatured, unable to bind toxin. Recent results on the electron microscopic visualization of the purest preparations revealed rod-shaped particles of 40 × 170 Å. Estimates of volume, together with the protein partial specific volume from the present amino acid composition (0.752 cm^3/g), suggested a molecular weight for these particles of the order of 257 000, or about one copy of the large peptide per rodlet (Ellisman et al., 1982).

We assume that the α-peptide subunit of the saxitoxin protein from synaptosomes (Hartshorne & Catterall, 1981) may be analogous to the large glycopeptide isolated here. This analogy suggests that this peptide ultimately may be shown to comprise gating structures as well. Whether peptides corresponding to the β-peptides exist in the electroplax, but have been lost due to dissociation or proteolysis, we cannot decide. Although it seems unlikely in view of the in situ labeling experiments with the arylazido *Leiurus* toxin (Beneski & Catterall, 1980), the β-peptides may be derived from the large peptide by partial proteolysis. We do not expect the electroplax protein to be fundamentally different from sodium channel peptides in the central nervous system because antibodies specific for this peptide labeled focally the nodal regions of myelinated and demyelinated nerves from the eel (Ellisman & Levinson, 1982). The large peptide apparently does not correspond to the peptides reported for the saxitoxin binding protein from sarcolemma (Barchi et al., 1980) although these workers have now detected a larger molecular weight species (R. Barchi, unpublished experiments).

Although it is possible to prepare smaller amounts of nearly homogeneous native protein, resolution of the bulk of the binding activity from the batch ion-exchange contaminants is incomplete; and two Sepharose 4B chromatographic runs are required for the preparation of low amounts (~0.10 mg). After denaturation in NaDodSO$_4$, however, the large peptide is essentially quantitatively resolved from other contaminants by chromatography over Sepharose 4B. By this means, it is possible to prepare 1.5–2 mg of the peptide per kg of electric organ.

The analysis of the peptide on NaDodSO$_4$–polyacrylamide gels leads us to conclude that (1) it is not dissociated to smaller species by even fairly harsh methods of denaturation, (2) it is a glycopeptide, (3) it is electrophoretically microheterogeneous, (4) it has an extremely high free electrophoretic mobility in NaDodSO$_4$, and (5) its molecular weight, while impossible to estimate reliably with this approach, is likely to exceed the 260 000 estimate reported earlier (Agnew et al., 1980, 1981).

The high free mobility of the peptide in $NaDodSO_4$ is not likely to be due to glycosylation. A conventional peptide would bind approximately 140% $NaDodSO_4$ by weight per g of protein (Reynolds & Tanford, 1970). For a 250 000-dalton species, this corresponds to approximately 2600 negative charges at pH 8. Even if sialic acid residues (11.8% by weight) represented a net charge, this would contribute only 104 negative charges, not enough to markedly alter mobility. Further, in well-characterized cases [cf. Pitt-Rivers & Impiombato (1968)], most glycopeptides bind proportionately less $NaDodSO_4$ than nonglycosylated species, lowering the free mobility rather than raising it. In keeping with this, the estimated molecular weights of glycopeptides usually decrease with increasing percentages of acrylamide, an obvious consequence of having a lower free mobility than that of the standards (this is apparent upon inspection of Figure 6). The peptide here, in contrast, appears to increase in molecular weight with increasing percentage of acrylamide from 3.5% to 5% acrylamide (Table III). (The molecular weight appears to then decrease between 5.5% and 6.5% acrylamide, but these are gel concentrations where extrapolated molecular weights for even well-behaved large peptides would be underestimated due to nonlinearity of the standard curve at extremes of mobility.) The explanation which we propose is that the peptide may bind large amounts of $NaDodSO_4$. There is no reason to anticipate such an effect on the basis of the amino acid composition, which indeed suggests a molecule somewhat less hydrophobic than the average intrinsic membrane protein. There may be, however, domains of the peptide which bind excessive amounts of amphipathic molecules, such as $NaDodSO_4$ or lipids (Agnew & Raftery, 1979; Robinson & Tanford, 1975). We have pointed out the peptide molecular weight may exceed 260 000. This cannot be assessed by methods such as gel filtration in $NaDodSO_4$ (Nozaki et al., 1976), because large peptides, even unglycosylated, are not well-behaved under these conditions. Less empirical methods, such an analytical ultracentrifugation, are preferable. The amino acid and carbohydrate compositions reported here have provided values for the partial specific volume ($\bar{v}$) of the peptide for these determinations.

Attempts have been made to devise somewhat empirical functions which would permit classification of proteins as soluble, peripheral, or intrinsic membrane proteins, based on the ratios of polar and hydrophobic amino acids [see Barrantes (1975) and Bigelow (1967)]. It is rationalized that polar residues would occupy predominantly the outer membrane surface, interacting with water, whereas hydrophobic moieties would be buried in the molecular interior (Bigelow, 1967). Proteins whose surfaces were in extensive contact with the hydrophobic bilayer would be enriched in surface hydrophobic residues. Of those parameters proposed, some have shown a degree of reliability in distinguishing between soluble and intrinsic membrane proteins. Table IV compares the values for the large peptide of two such parameters with those in other electroplax proteins. By the $H\phi_{av}$ (Bigelow, 1967), the TTX binding peptide is in the more polar range of membrane proteins and in the more hydrophobic range of soluble proteins. By the z parameter (Barrantes, 1975), however, the peptide is even more clearly intermediate between the two classes despite the fact that this parameter is considered to be more discriminating between the intrinsic and peripheral classes of protein. This is somewhat surprising in that it is clear that the TTX binding protein is an intrinsic membrane protein displaying readily observed interactions with membrane lipids (Agnew & Raftery, 1979). It also suggests that there is nothing extreme about the peptide's overall composition which would predict the binding of unusual amounts of negatively charged $NaDodSO_4$ which would be required to account for the unusual mobility during electrophoresis.

The relatively large amount of carbohydrate enriched in sialic acid deserves two specific comments. The native TTX binding protein appears to be highly charged at neutral pH. This is evidenced by its adsorption to DEAE ion-exchange resins in the presence of high salt and acidic polyelectrolytes (Agnew et al., 1978), and in its high electrophoretic mobility on nondenaturing agarose gels (Agnew et al., 1978, 1981). The latter behavior allows us to estimate that the TTX binding protein may contain 6 times as many negative charges as the moderately acidic acetylcholine receptor (S. R. Levinson and W. S. Agnew, unpublished experiments). The proportion of sialic acid found may help account for the acidity of the native molecule.

In addition, there may be a functional role for the sialic acid residues. It has been shown that placement of a single negative charge near the opening of the pore-forming antibiotic gramicidin is sufficient to raise the cation conductances at least 3-fold over that of underivatized gramicidin A (Apell et al., 1977). Furthermore, evidence exists for a conductance-increasing anionic group near the mouth of the sodium channel (Sigworth & Spalding, 1980). Because glycosylation of membrane proteins is, as far as known, always on the external side of the membrane, we suggest that the sialic acid residues may contribute to enhanced inward flow (i.e., conductance) of sodium ions through the channel. If true, this glycosylation may be a general feature of sodium channels or, alternatively, may be a specialization of the electroplax sodium channel to produce a greater discharge from the electric organ. It will be interesting to see whether the α-peptide of the sodium channel from synaptosomes is glycosylated to a similar extent. As discussed above, the electrophoretic microheterogeneity of the peptide may well result from steric effects of carbohydrate moieties. Such behavior has been observed with other glycopeptides (Horowitz, 1977).

It is anticipated that the ion-transporting channel may be associated with the large peptide characterized here, but it will be of interest to see whether this function, together with the complex gating mechanisms, will be displayed by reconstituted preparations containing only this component.

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Ordered Phosphorylation of the Two 20 000 Molecular Weight Light Chains of Smooth Muscle Myosin†

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ABSTRACT: The time courses of phosphorylation of the M_r 20 000 light chains by purified myosin light chain kinase plus calmodulin were determined. In confirmation of an earlier report [Persechini, A., & Hartshorne, D. J. (1981) *Science (Washington, D.C.) 213*, 1383–1385], a steady-state kinetic analysis indicates that the phosphorylation occurs in an ordered manner; i.e., at a phosphorylation level of 0.5 mol of ^{32}P incorporated per mol of bound M_r 20 000 light chain, each myosin molecule would have one phosphorylated head. The kinetic parameters obtained for the phosphorylation of the more reactive myosin head are similar to those determined by using isolated light chains. It is suggested that the ordered, or sequential, phosphorylation, and the different reactivities of the two M_r 20 000 light chains, is the result of preexisting asymmetry of the myosin molecule. Similar patterns of myosin phosphorylation are obtained in both the absence and presence of skeletal muscle actin.

One of the theories which has been proposed to account for the activation of the contractile apparatus in smooth muscle is based on the phosphorylation of the myosin molecule. Phosphorylation of the M_r 20 000 light chains is thought to allow activation by actin of the Mg^{2+}-ATPase activity of myosin, whereas in the dephosphorylated state myosin is not activated by actin [see reviews by Adelstein & Eisenberg (1980) and Hartshorne & Mrwa (1982)]. Although this theory has received much support, it is not accepted universally [cf. Nonomura & Ebashi (1980)], and unequivocal evidence that myosin phosphorylation–dephosphorylation forms the dominant regulatory mechanism is still to be presented. Consequently, each phase of the phosphorylation scheme continues to be analyzed, and this includes the phosphorylation reaction which obviously is obligatory for subsequent phos-

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