Vol. 92, No. 3, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 12, 1980 Pages 860-866

IDENTIFICATION OF A LARGE MOLECULAR WEIGHT PEPTIDE ASSOCIATED WITH A TETRODOTOXIN BINDING PROTEIN FROM THE ELECTROPLAX OF ELECTROPHORUS ELECTRICUS

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Received December 19,1979

SUMMARY: We have previously described the purification of a tetrodotoxin binding protein from the electroplax of Electrophorus electricus. The preparation consisted of three peptides of M_r ~ 46,000, 59,000 and ~ 300,000 daltons. Further investigation has now shown that the large peptide of M_r ~ 260,000 daltons is part of the tetrodotoxin binding component of the voltage-sensitive sodium channel.

INTRODUCTION

We have previously described the solubilization and purification of a TTX[1] and STX binding protein which is associated with the voltage-sensitive sodium conductance channel in the electroplax of Electrophorus electricus, (1). The most highly purified material had specific activities of over 2000 pmol [^{3}H] TTX binding/mg protein and, by SDS polyacrylamide gel electrophoresis, consisted of three peptides of M_r ~ 300,000, 59,000 and 46,000 daltons. The purification reported included the preparation of membranes, solubilization with Lubrol-PX, fractionation by a batch ion-exchange procedure with DEAE Sephadex A-25, and two successive elutions through columns of Sepharose 6B.

We have found that ion-exchange purified samples were generally of high specific activities (250-500 pmol TTX bound/mg protein) and of simple and reproducible peptide composition. Subsequent fractionation by a single step

Supported by UPHS Grant NS-12018 and by a grant from the American Heart Foundation, Greater Los Angeles Chapter and N.I.H. Postdoctoral Fellowships to W.S.A., A.C.M. and S.R.L..
Contribution # 6151

[1]Abbreviations used: EDTA, ethylenediamine tetra-acetic acid; PMSF, phenylmethane sulfonyl fluoride; SDS, sodium dodecyl sulfate; TTX, tetrodotoxin; STX, saxitoxin

0006-291X/80/030860-07$01.00/0

of gel exclusion chromatography, native gel electrophoresis, or sucrose gradient sedimentation yielded preparations with specific activities usually in the range of 1300-1800 pmol/mg protein. In these samples the major component was a large peptide of M_r ~ 260,000 daltons. It was also possible to examine the correspondence between the distribution of individual peptides and that of [^{3}H] TTX binding activity in each of the fractionated profiles. In every instance it was found that the large peptide correlated with [^{3}H] TTX binding activity. Other peptides, present in lesser and variable amounts in fractions of high specific activity, showed no clear covariance with binding activity.

MATERIALS AND METHODS

Procedures for the preparation of [^{3}H] TTX (for these studies, two samples of 54.5 and 78.8 $\mu C_i/\mu mol$), and for [^{3}H] TTX binding have been described elsewhere (1,3,8).

The main electric organ was removed from small eels (1 m), and stored at -90°C. Subsequently the partially thawed tissue was diced into cubes of approximately 1 cm^3 and suspended in 3-5 volumes of cold 0.05 M potassium phosphate buffer, with two one minute cycles with the large head (3.5 cm) of the Polytron tissue disrupter and two one minute cycles with the small head (2.5 cm). The homogenate was centrifuged at 25,000 xg for 1 hour in the Sorvall GSA rotor. The supernatant was discarded and the pellet resuspended with the Polytron large head and recentrifuged. The pellet was resuspended in fresh buffer, and packed by centrifugation at 100,000 xg for 1 hour in a Beckman 42.1 rotor. Packed membranes were removed, weighed and resuspended in 2 ml/g (w/w) of 0.05 M potassium phosphate buffer, pH 7.4, 0.1 mM EDTA, 0.1 mM PMSF. Aliquots of the membrane suspension were stored frozen at -80°C.

Batch-concentrates were prepared as described by Agnew et al (1) except that buffers contained 0.1 mM EDTA and 0.1 mm PMSF, and adsorbtion and desorption was with 0.2 M and 0.5 M KCl rather than NaCl.

Sepharose 6B chromatography of batch-purified material was performed as described earlier (1) except the column was 1.5 × 100 cm. For the experiment illustrated in Figure 3, a sample of concentrate (2890 pmol [^{3}H] TTX sites in 2.4 ml) was delivered to the column, which was eluted at 11 ml/hr; 3.3 ml fractions were collected. Recovery of activity was 81% of that applied. Fractions were assayed for [^{3}H] TTX binding and for protein (13). Aliquots of neighboring fractions were pooled, concentrated and run on SDS polyacrylamide gels.

Native gel electrophoresis was performed on 1% agarose slabs (Marine Colloids, Inc.) containing 0.05 M, potassium phosphate buffer, pH 7.45, 20 mM [^{3}H] TTX, 0.1% (w/v) Lubrol-PX and phosphatidyl choline (7:1 molar ratio). The gel was 17 × 3.5 × 0.5 cm. The sample was batch-purified material, 463 pmol in 0.5 ml, 367 pmol/mg. It was run for 7 hours at 65 volts and 95 mA on a Desaga flatbed gel apparatus, at 3°C. After the run, 0.43 cm wide slices of the gel were placed in a cold 12 ml Sorvall tube and centrifuged at 48,000 g in the SS 34 rotor for 10 min. The supernatants were assayed for [^{3}H] TTX binding protein and analyzed by SDS polyacrylamide gel electrophoresis. The recovery of TTX binding activity was 72% of that applied.

The sucrose gradient fraction was carried out in a 34 ml gradient, 5-20% sucrose, containing 0.1% of a 7:1 molar ratio of Lubrol-phosphatidyl choline, 0.05 M potassium phosphate, pH 7.5, 20 nM [^{3}H] TTX, in the VTi 50 reorienting rotor. A sample of concentrate (403 pmol/mg protein, 1920 pmol in 1.8 ml) was layered over the gradient and centrifuged at 48,000 rpm for 3.5 hours. Fraction (0.7 ml) were removed and assayed for [^{3}H] TTX binding and protein. Aliquots of neighboring fractions were pooled, concentrated and analyzed by SDS-polyacrylamide gel electrophoresis. Recovery of activity was about 49% of that applied.

SDS polyacrylamide gel electrophoresis was by the method of Laemmli (7). The running gel contained 6% (w/v) polyacrylamide, 0.125% (w/v) N, N'-methylene bis acrylamide. After staining with Coomassie Brilliant Blue, the gels were scanned with the linear transport attachment of a Gilford 240 spectrophotometer at 650 nM. The area under the peaks for individual peptides was taken as a semi-quantitative measure of the amount of that peptide present.

RESULTS

Figure 1A illustrates the typical peptide composition of batch-purified material. There were nominally seven peptides, labeled 1 through 6, of

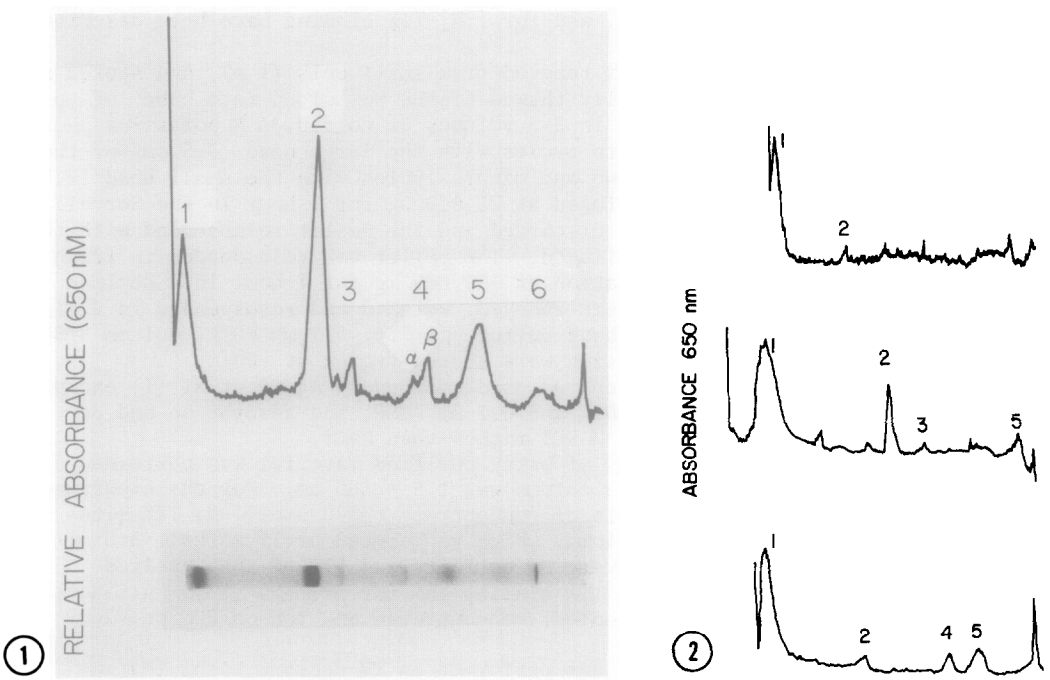
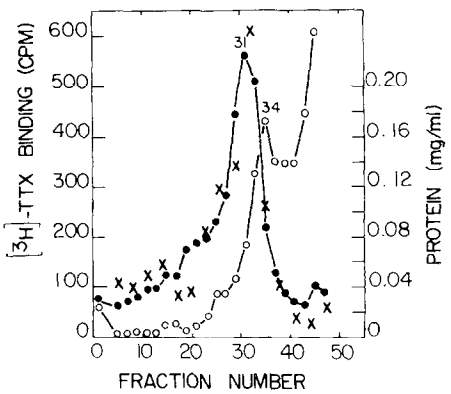


FIGURE 1: SDS-polyacrylamide gel and densitometer scan for a sample of batch concentrate (403 pmol/mg protein): Peptides labeled 1-6 have the molecular weights given in the text.

FIGURE 2: Densitometer scans of gels run on fractions of peak specific activity after fractionation of batch concentrate by (TOP) sucrose gradient sedimentation (gel 25-27, Figure 3); (MIDDLE) native gel electrophoresis (fraction 8, Figure 5); (BOTTOM) Sepharose 6B column chromatography (gel 29-30, Figure 4).



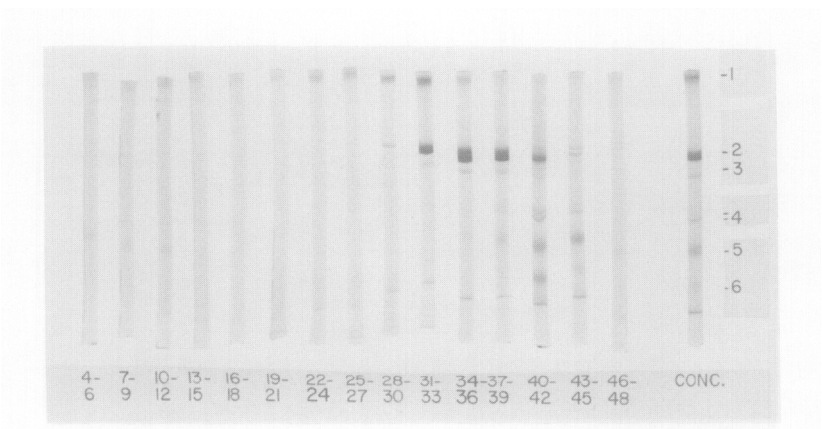
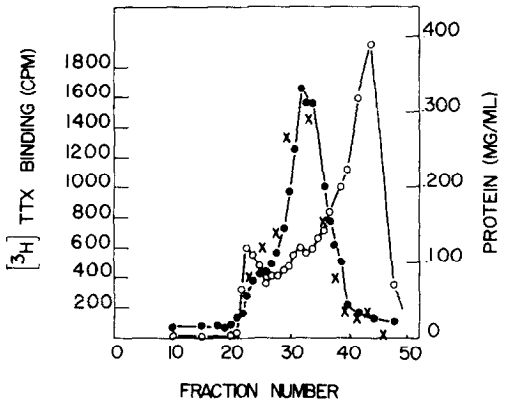


FIGURE 3: Sucrose density gradient profile: (○) protein by fluorescamine, (●) [^{3}H] TTX binding; (✕) relative area under densitometer scans of band 1, measured on the gels shown. SDS gels were run on equal volumes of pooled aliquots from neighboring fractions, as indicated.

M_r ~ 260,000, 95,000, 79,000, 52-59,000, 42,000 and 34,000 daltons respectively The peptides labeled 4 α,β were sometimes represented by 1 or 3 bands, and band 6 was sometimes absent.

Figure 2 shows densitometer scans of gels run on fractions of peak specific activity after batch purified material was subjected to sucrose gradient sedimentation (1680 pmol/mg protein), native gel electrophoresis (1331 pmol/mg protein) and after one elution from Sepharose 6B (1170 pmol/mg protein). The major component of each purified sample was the ~260,000 dalton peptide. Other bands, notably those of 95,000, 59,000 and 42,000 were present in lesser and variable amounts.



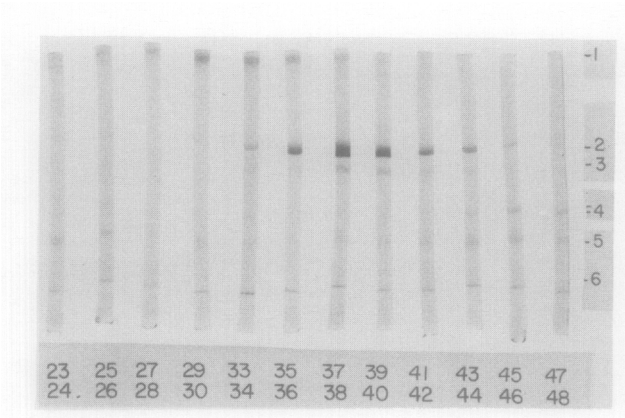


FIGURE 4: Sepharose 6B column profile: symbols are as in Figure 3. Gels were performed on pooled aliquots from neighboring fractions as indicated.

Inspection of the SDS gel patterns of fractions collected from a column (Figure 3), sucrose gradient (Figure 4), or native gel (gels not shown) revealed a close correlation between [^{3}H] TTX binding activity and the distribution of band 1. In the experiment shown in Figure 3, a sample of batch concentrate was sedimented through a 5-20% sucrose gradient. At the top of the gradient there was a large peak of fluorescamine positive material which contained, however, few peptides. Amino acid analysis suggests this may not be protein . The major peak of protein was just resolved from the peak of TTX binding activity. The profile for TTX binding was slightly skewed, suggesting a tendency to form higher molecular weight aggregates. The gels show that only band 1 clearly correlated with toxin binding activity, with a similar

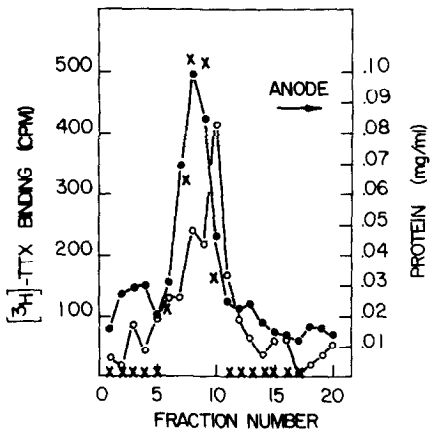


FIGURE 5: Native starch gel electrophoresis profile: symbols are as in Figure 3. SDS gels were run on aliquots taken from each fraction. The scan in Figure 2 (MIDDLE) is of the gel from fraction 8.

skew in the distribution of band 1 towards the bottom of the gradient. The pattern of distribution of the 95,000 dalton peptides suggests that they may represent contaminants in fractions of highest specific activity.

In Figure 4 results are shown of the elution profile from a column of Sepharose 6B. Again, inspection of the gels revealed that only the distribution of band 1 correlated clearly with the distribution of toxin binding activity. Peptides of 95,000, 79,000, ~57,000 and 34,000 were clearly resolved from the peak of TTX binding activity. From the trends, it cannot be decided whether peptides of 59,000 and 42,000 daltons contribute to the TTX binding protein, but it again seems likely that the 95,000 dalton species may represent a contaminant.

Fractionation of batch-concentrates by native gel electrophoresis revealed the same general pattern of fractionation. Again, only the large peptide (Figure 2, middle) was clearly correlated with the distribution of TTX binding activity (Figure 5). Despite a much greater Stokes' radius (95Å), the solubilized TTX binding molecule had roughly the same mobility as the nicotinic acetylcholine receptor from Torpedo californica (67Å) (11). This suggests that the TTX binding component carries a substantial net negative charge at neutral pH.

DISCUSSION

The results from these and related studies strongly indicate that the TTX and STX binding component is an acidic macromolecule composed at least in part of 260,000 dalton peptide(s). Other peptides are characteristically present in purified samples in lesser and in variable amounts. Because these, or other undetected peptides may be lost during solubilization and isolation, or may be especially sensitive to proteolysis, it cannot be decided whether the large peptide(s) constitutes the entire native sodium channel assembly. In addition, the distinct possibility that the ~260,000 dalton peptide(s) may exist in oligomeric association in the native molecule suggests that the molecular weight may be greater than the 240,000 previously estimated (9,1).

The functions and regulation of the sodium channel are remarkably complex and TTX and STX interfere only with the ion transport mechanisms, without affecting the voltage-sensitive gating processes (2,6,12). It will be of interest to see whether other neurotoxins which primarily alter channel gating (10,4,5), also bind to these or to other peptides present in the native molecule.

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