SOLUBILIZATION AND PARTIAL CHARACTERIZATION OF THE TETRODOTOXIN BINDING COMPONENT FROM NERVE AXONS*

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Summary

The tetrodotoxin binding component from garfish olfactory nerve membranes has been solubilized using the nonionic detergent Triton X-100. Tetrodotoxin binds to the solubilized component with a dissociation constant $K_D = 2.5 \times 10^{-9}$ M and under saturating conditions 1.95×10^{-12} moles of tetrodotoxin are bound per milligram of solubilized protein. Upon solubilization the toxin binding component becomes much less stable towards heat, chemical modification and enzymatic degradation. Sucrose gradient velocity sedimentation yields an S value of 9.2 for the extracted binding component and from gel filtration data the binding component appears to be slightly larger than β-D-galactosidase.

The physiological specificity of tetrodotoxin has been well established (1); it acts selectively and at extremely low concentrations to inhibit the flux of sodium ions through the sodium channels in most nerve axons by a presumed tight binding to the channel (2). Other molecular components of the nervous system including potassium channels, the sodium-potassium activated ATPase, and post-synaptic receptors are unaffected by tetrodotoxin (3-5).

We have previously exploited this great specificity of tetrodotoxin by using tritiated tetrodotoxin as a marker of sodium channels in nerve membranes (6). We report here some biochemical properties

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of the detergent-solubilized tetrodotoxin binding component including Stokes radius from gel filtration columns, S value from sucrose gradient velocity sedimentation, and susceptibility towards enzymatic degradation.

Experimental

Materials: Tritiated tetrodotoxin was prepared as described earlier (6). Frozen Lepisosteus osseus heads were obtained from E. Saeugling (Guttenberg, Iowa). Enzymes used were those previously described (6). All chemicals were reagent grade except where specified.

Preparation of Triton Extract: Membrane fragments from the olfactory nerve were prepared as described previously (6). The membrane pellet was suspended in the extraction medium, consisting of garfish physiological solution (7) which had been made 10% in glycerol and 5% in Triton X-100. The membranes were extracted with stirring for 10-12 hours at 4° C and centrifuged at 100,00 x g for 1 hour in an L-3-50 Beckman Ultracentrifuge. The supernatant was decanted and is subsequently referred to as the Triton extract, which was kept below 10° C at all times.

Binding Assay: Equilibrium dialysis binding studies were performed as previously described (6).

Enzyme Treatment and Chemical Modifications: For each enzyme treatment, 0.35 ml of Triton extract was incubated with 0.5 mg of the various enzymes (except Phospholipase A which was 0.1 mg per assay) for 10 hours at 4° C. The treated Triton extract was then placed in dialysis tubing and the equilibrium dialysis assay performed.

Molecular Weight Determinations: Sucrose gradient (5-20%) velocity sedimentation was performed according to the method of Martin and Ames (8). The gradients were made up in the Triton extraction solution and 4.0 nM [^{3}H] tetrodotoxin was distributed uniformly throughout the gradient. A 200 µl sample was layered on the preformed gradients and centrifuged for 10 hours at 65,000 rpm in a SW65K rotor in a L-2-65B Beckman Ultracentrifuge.

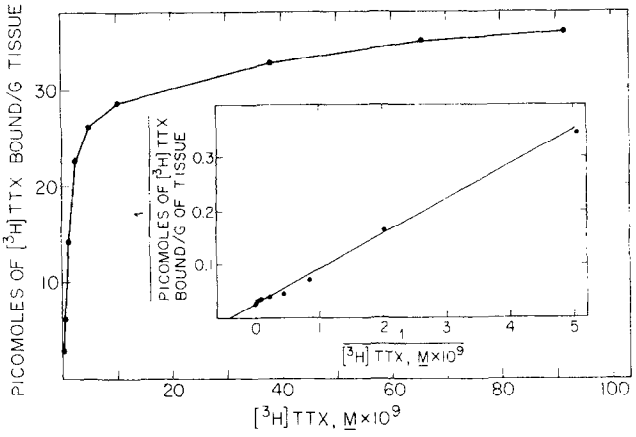


Figure 1: Binding curve of [^{3}H]tetrodotoxin to the Triton extract of axon membranes by equilibrium dialysis. Picomoles of [^{3}H]tetrodotoxin bound per gram of tissue is plotted against the concentration of free [^{3}H]tetrodotoxin. The insert shows the reciprocal plot of the binding data.

A Sepharose 6B column (1.5 x 98 cm) was equilibrated in Triton extraction buffer containing 0.5 nM [^{3}H] tetrodotoxin. A 1 ml sample of Triton extract was applied, and the amount of radioactivity measured in the effluent, according to Hummel and Dreyer (9). This procedure was also used for sucrose gradients.

Results and Discussion

Solubilization: By use of 5% Triton X-100 solutions it was possible to extract greater than 90% of the tetrodotoxin binding activity from membranes. 10% glycerol was used to stabilize the solubilized component.

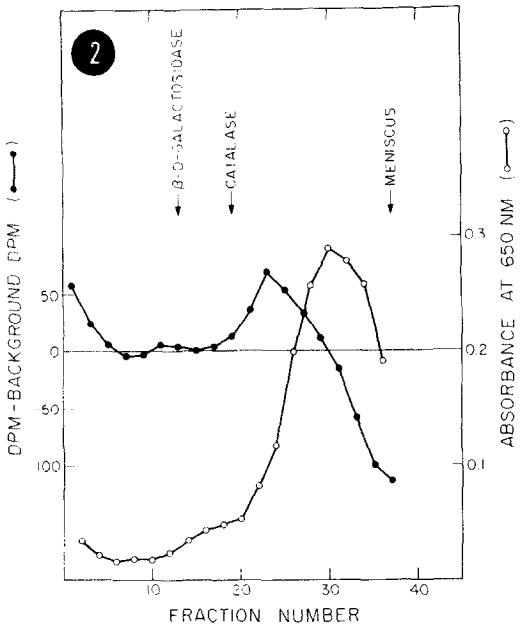
The binding curve obtained for the Triton-solubilized material (Fig. 1) shows a single binding site with a dissociation constant $K_D = 2.5 \times 10^{-9}$ M. Under saturating conditions there are 39 picomoles of toxin bound per gram of wet tissue or 1.95 picomoles of toxin per milligram of solubilized protein as measured by the Lowry method (10). It is interesting to note that the affinity of tetrodotoxin for the binding component is increased upon solubilization from $K_D = 8.3 \times 10^{-9}$ M obtained from membrane fragments (6). This phenomenon is possibly due

Table 1: Effects of Enzyme Treatment and Chemical Modification on Tetrodotoxin Binding in Triton Extracts

Treatment	Fraction of Binding Activity Remaining After Treatment
Ribonuclease	0.87
Deoxyribonuclease	0.87
Hyaluronidase	0.92
Neuraminidase	1.02
Phospholipase A	0.23
Phospholipase C	0.96
Trypsin	0.07
α-Chymotrypsin	0.25
Pronase	0.11
Heat 25° C (1 hour)	0.15
DTT 5 mM	0.12
ICH_2CONH_2 5 mM	0.97

to increased accessibility of the toxin binding site in the solubilized material.

Enzyme Treatments and Chemical Modifications: The most striking finding (Table 1) is the decreased stability of the tetrodotoxin binding component when it is extracted from the membrane. The Triton extract is quite sensitive to trypsin, chymotrypsin and pronase, whereas the binding component is relatively insensitive towards proteolytic digestion in the membranes (6). The solubilized binding component is unstable at room temperature for any length of time so that all procedures must be performed below 10° C. At 0-4° C, however, it is stable for several days. Apparently the binding component contains at least one disulfide bond necessary for binding activity which is partially protected against reduction when the component is in the membrane (T. Benzer and M. Raftery, unpublished data). The solubilized binding component retains a phospholipid requirement as seen by its sensitivity towards inactivation



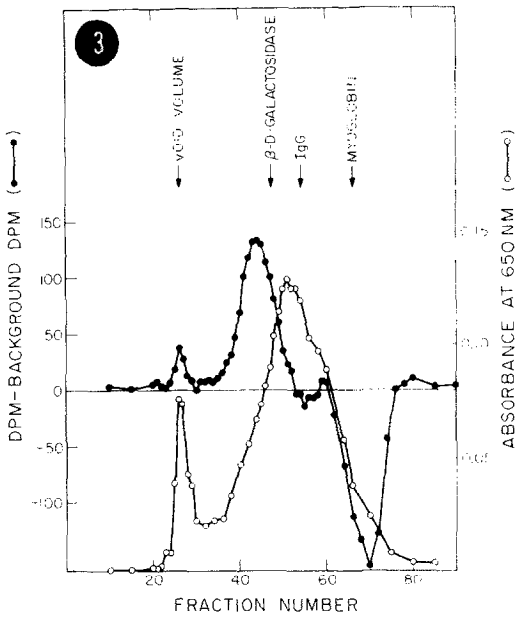


Figure 2: Sucrose gradient sedimentation of the Triton extract. β-D-galactosidase ($S_{20,w}$ = 16) and catalase ($S_{20,w}$ = 11.4) as marker proteins and the sample were run simultaneously. 140 μl fractions were collected and 100 μl aliquots were assayed for radioactivity and protein (10). The radioactivity measurements were corrected for the low level of [^{3}H]tetrodotoxin (269 dpm/100 μl) found throughout the preformed gradient.

Figure 3: Sepharose 6B chromatography of 1 ml of Triton extract. The column was calibrated using β-D-galactosidase, IgG and myoglobin. The fraction size was 2.0 ml. 1.0 ml aliquots were assayed for radioactivity and 100 μl for protein determination. Radioactivity was corrected for the constant background of [^{3}H] tetrodotoxin (345 dpm/ml) in the elution buffer.

by Phospholipase A. It is possible that the solubilized binding component retains a certain amount of associated phospholipid which is necessary for binding activity, even in the presence of Triton X-100.

Molecular Weight Determinations: The sucrose gradient results show that the binding component sediments slightly slower than catalase with an apparent S value of 9.2 (Fig. 2). There is also a fast sedimenting peak at the bottom of the centrifuge tube which is presumably due to aggregation or incomplete solubilization of the binding component. In contrast to its behavior on sucrose gradients, the binding component appears

slightly larger than β-D-galactosidase in the Sepharose 6B elution profile (Fig. 3). Similar results have also been reported by Henderson and Wang (11). The discrepancy in size estimation between sucrose gradients and gel filtration could be due to many factors including the shape, amount of associated lipid, and amount of bound detergent.

Similar discrepancies in size between sucrose gradient and gel filtration data have been observed for detergent extracts of the acetylcholine receptor from electric fish (12). Upon effective solubilization of the tetrodotoxin binding component in active form, it now becomes feasible to continue its characterization and eventual purification.

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