Affinity Purification of the Voltage-Sensitive Sodium Channel from Electroplax with Resins Selective for Sialic Acid†

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Received September 28, 1988; Revised Manuscript Received March 9, 1989

ABSTRACT: The voltage-sensitive sodium channel present in the eel (*Electrophorus electricus*) has an unusually high content of sialic acid, including α-(2→8)-linked polysialic acid, not found in other electroplax membrane glycopeptides. Lectins from *Limax flavus* (LFA) and wheat germ (WGA) proved the most effective of 11 lectin resins tried. The most selective resin was prepared from IgM antibodies against *Neisseria meningitidis* α-(2→8)-polysialic acid which were affinity purified and coupled to Sepharose 4B. The sodium channel was found to bind to WGA, LFA, and IgM resins and was readily eluted with the appropriate soluble carbohydrates. Experiments with LFA and IgM resins demonstrated binding and unbinding rates and displacement kinetics, which suggest highly specific binding at multiple sites on the sodium channel protein. In preparative-scale purification of protein previously fractionated by anion-exchange chromatography, without stabilizing TTX, high yields were reproducibly obtained. Further, when detergent extracts were prepared from electroplax membranes fractionated by low-speed sedimentation, a single step over the IgM resin provided a 70-fold purification, yielding specific activities of 3200 pmol of [^{3}H]TTX-binding sites/mg of protein and a single polypeptide of ~285 000 Da on SDS–acrylamide gels. No small peptides were observed after this 5-h isolation. We further describe a cation-dependent stabilization with millimolar levels of monovalent and micromolar levels of divalent species.

Voltage-sensitive Na channels are large integral membrane proteins responsible for the propagation of the action potential in many excitable cells. They form a sodium-selective pathway across electrically excitable cell membranes in a transient response to changes in transmembrane voltage. The main electric organ of the eel, *Electrophorus electricus*, is capable of generating electrical discharges of more than 1000 V. It is perhaps the richest preparative source of a TTX-sensitive sodium channel protein and is the first tissue from which sodium channels were isolated (Agnew et al., 1978). To better exploit this preparation, we have developed new methods with which to purify rapidly large quantities of undenatured protein, suitable for functional reconstitution, chemical modification, or visualization by electron microscopy.

The electroplax channel is a large polypeptide of 208 321 Da (Miller et al., 1983; Noda et al., 1984), to which is attached approximately 85 000 Da of carbohydrate. We have distinguished previously two major kinds of N-linked oligosaccharides in deglycosylation studies with various endoglycosidases. These chains, apparently linked via one- or two-armed cores, include a neutral, high mannose or hybrid class and an acidic, complex class containing sialic acid [for reviews, see Farquahar (1985) and Roth (1987)]. A surprising discovery was that much of the sialic acid is present as extended unbranched homopolymers of α-(2→8)-linked polysialic acid, evidently extending >10 nm from the protein surface (James & Agnew, 1987, 1988b, 1989b). The sodium channel appears to be the only protein bearing this carbohydrate in the electroplax (James & Agnew, 1989b).

These findings suggested a rationale for affinity purification for convenient and efficient isolation from the enriched electroplax membranes. Initial detergent extracts contain 15–30 pmol of TTX-binding sites/mg of protein, about 0.5% that expected for pure, native protein (Agnew et al., 1978). DEAE ion exchange produces a 40–50-fold increase in specific activity, with 60–70% recovery of binding sites (Agnew et al., 1978; Duch & Levinson, 1987). One or two steps of gel filtration, performed in the presence of stabilizing [^{3}H]TTX and mixed lipid/detergent micelles, yielded final purification, with some fractions incompletely resolved from contaminants (Miller et al., 1983). These methods present serious disadvantages. Denaturation during ion exchange cannot be avoided, stabilizing TTX interferes with functional reconstitution studies, and pressure concentration before and after gel filtration is slow and elevates the concentration of detergent and lipid. The unusual oligosaccharides of the protein suggested that rapid and selective affinity methods could be devised that avoid these problems.

We examined 11 lectin-affinity resins, together with a resin made from specific immunoglobulins raised against bacterial polysialic acid. We found the electroplax sodium channel does bind tightly to WGA resins having a high WGA content, although this lectin does not result in optimal purification. The high-affinity, sialic acid specific lectin from *Limax flavus* (LFA) binds the sodium channel avidly and can be used in efficient purification protocols subsequent to DEAE fractionation. In addition, we purified, by affinity chromatography, antibodies raised against bacterial capsular α-(2→8)-polysialic acid. In the electroplax, these antibodies bind only to sodium channel. Affinity resins prepared with these antibodies avidly adsorb the protein, which can be subsequently eluted by adding bacterial antigen with nearly quantitative recovery. We further describe the stabilizing effects of low concentrations of monovalent and divalent cations. The affinity procedures reported do not require stabilizing neurotoxins and can be carried out in a few hours. The immune-affinity resin, used in a one-step purification, yields protein that is essentially homogeneous by SDS–PAGE, with maximum specific activities of 3200 pmol of TTX-binding sites/mg of

† This research was supported by NIH Grants NS 17928 and HC 38156 and a grant from the National Multiple Sclerosis Society to W.S.A. and by NIH Postdoctoral Fellowship NS07102-09 to W.M.J.
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0006-2960/89/0428-6001$01.50/0

protein. A preliminary account of some of these findings has been presented (James & Agnew, 1988a,b, 1989a).

Materials and Methods

Materials

Lectins from jack bean (*Canavalia ensiformis*), horseshoe crab (*Limulus polyphemus*), red kidney bean E and L (*Phaseolus vulgaris*), pea (*Pisum sativum*), castor bean (*Ricinus communis*), and potato (*Solanum tuberosum*) were obtained from Sigma. Lentil (*Lens culinaris*) and jackfruit (*Artocarpus integrifolia*) lectins were from Vector Labs. Pure wheat germ (*Triticum vulgaris*) and slug (*Limax flavus*) lectins were obtained from E. Y. Labs, and in some experiments the latter was affinity-purified, from the crude grade, against mucin–agarose exactly as described by Miller (1987). Horse antiserum raised against capsular α-(2→8)-polysialic acid of *Neisseria meningitidis* was the kind gift from J. B. Robbins (NIH). Citrate-free TTX was the kind gift of Y. Kishi, Harvard University, and it was tritiated at New England Nuclear by the Wilzbach procedure and purified as described (Agnew et al., 1978). The toxin was originally of specific activity ~70 Ci/mmol (1 Ci = 37 GBq) and ≈65% radiochemical purity as determined by frog sciatic nerve bioassay. Mucin, CNBr-activated Sepharose 4B, Lubrol-PX, egg phosphatidylcholine (type IX-E), *N*-acetylglucosamine, *N*-acetylneuraminic acid from *Escherichia coli* (type VI), colominic acid, and polyacrylohydrazido-agarose (4% beaded, 16 μmol/mL) were from Sigma.

Methods

Purification of TTX-Binding Protein. Washed crude membranes were prepared, and a Lubrol-PX extract of this was fractionated on DEAE-Sephadex, exactly as previously described by Rosenberg et al. (1984a). The pooled DEAE protein (DP) was obtained from this in buffer A (0.4 M KCl, 0.05 M sodium phosphate, pH 7.4, and protease inhibitors: 50 μM *o*-phenanthroline, 50 μM L-1-(tosylamido)-2-phenylethyl chloromethyl ketone, 100 μM phenylmethanesulfonyl fluoride, 1.0 mM EGTA, and pepstatin A at 0.1 μg/mL). [^{3}H]TTX binding was measured by the Sephadex G-50 assay as previously described (Agnew et al., 1978; Levinson et al., 1979). Protein was measured either with fluorescamine (Böhlen et al., 1973) or Coomassie dye (Bradford, 1978). This preparation was used where indicated below.

In some experiments with IgM immunoresin, the previous method of membrane fractionation was modified as follows. Minced electric organ was homogenized (Polytron) in 5 volumes (w/v) of buffer (50 mM sodium phosphate, pH 7.4, 5 mM EDTA, protease inhibitors as previously described at 50% dilution; all steps on ice). The homogenate was centrifuged at 4000*g* for 20 min, and the resulting pellet was resuspended in 66% of the first buffer volume and recentrifuged, giving fractions S_1, S_2, and P_2. Detergent extracts of these fractions were made in 1% Lubrol-PX with protease inhibitors; fraction P_2 was found to retain up to 90% of the original activity and only 56% of the protein. Material discarded (S_1 and S_2) had low specific activity (~6 pmol/mg of protein). Membrane detergent extract of P_2 having a specific activity ~45 pmol/mL was applied directly to an IgM immunoresin.

Preparation of Polysialosylagarose Resin. Terminal residues of colominic acid [α-(2→8)-linked polysialic acid from *E. coli* strain K1 coat polysaccharide] were first oxidized as described by Jennings and Lugowski (1981). Colominic acid was rapidly dissolved to a 1% (w/v) solution in a 0.1 M solution of sodium metaperiodate freshly prepared in sodium acetate buffer, 100 mM at pH 5.5. This was reacted in the dark at room temperature for 15 min. To quench unreacted oxidizer, the solution was chilled on ice and a 1.0 M solution of sodium sulfite was added to make a final concentration of 200 mM. After 5 min, the solution was warmed to room temperature. Oxidized colominic acid was conjugated to polyacrylohydrazido-agarose equilibrated in PBS by mixing overnight at room temperature in a ratio of ~1 mg of oxidized colominic acid/mL of resin, at a scale of 25 mL of resin. Before use, the resin was washed with 20 volumes of PBS at 22 °C and then with 3 volumes of PBS at 45 °C.

Purification of Specific Antibody. Horse serum was centrifuged (6000*g* for 1 h) to remove precipitate. A mixture of 25 mL of colominic acid saturated agarose resin and 50 mL of diluted (1:1; PBS) serum was agitated for ≥1.5 h on ice. Unbound solutes were eluted by washing with 100 mL of PBS. In experiments to show salt destabilization of the binding, aliquots of resin already equilibrated with antibody and washed were pelleted in microfuge tubes and incubated with the indicated concentrations of NaSCN or $CaCl_2$ at 4 °C. Aliquots of the supernatants were assayed for protein. Thermal destabilization was similarly measured in individual small incubations. In routine preparative-scale purifications, bound antibody was desorbed by warming the resin in a jacketed column to 50 °C and applying several volumes of PBS at 50 °C. Protein was measured either by monitoring OD_{280} or by fluorescence assay using bovine IgG as standard.

Preparation of Protein–Agarose Resins. Proteins were concentrated in a pressure ultrafiltration cell (Amicon) over XM-50 or PM-10 filters (30 psi). Lectin, antibody, or mucin resins were synthesized at 4 °C by swelling and rinsing CNBr-activated Sepharose 4B in 1 mM HCl (200 mL/g of dry resin) and then rinsing in coupling buffer (0.5 M NaCl and 0.1 M $NaHCO_3$, pH 8.3), followed by incubation with protein (5–10 mg/mL of resin) overnight. Remaining reactive sites were blocked with 0.2 M glycylglycine ethyl ester at pH 8.0 for 2 h at room temperature, and resins were washed with 20 volumes of coupling buffer at 4 °C and then with 20 volumes of sodium phosphate buffer, pH 7.4, and 0.1% (w/v) Lubrol-PX at 23 °C.

Kinetic Studies. Two formats were used to measure the rate of binding to LFA–Sepharose. In the first, the resin was suspended in 3 volumes of buffer A and 0.35-mL aliquots were delivered to 0.5-mL microcentrifuge tubes. To these was added 0.1 mL of DP containing 35 pmol of Na channels, and samples were mixed end-over-end until assay at the times indicated in Figure 2A. Then, following a brief spin in a microcentrifuge, 235-μL aliquots of supernatant were added to 15 μL of [^{3}H]TTX, and after 10 min, samples were assayed in Sephadex G-50 columns as described. In an alternative format, 1.0 mL of Na channel (DP), diluted with 1.4 mL of buffer A, was mixed with 0.9 mL of resin equilibrated with buffer A and then immediately poured in a small column. The solution was recirculated with a peristaltic pump. Aliquots of 0.1 mL were removed and assayed as described at the indicated times. In both experiments, a background signal of 35 cpm was subtracted; the initial levels of [^{3}H]TTX binding were respectively 1144 and 235 cpm. To measure the rate of dissociation from LFA–Sepharose, 2.0 mL of the resin was equilibrated with ~1100 pmol of [^{3}H]TTX sites for 60 min, and then the resin was rinsed with 10 mL of buffer A five times. Three volumes of buffer A was added, and 0.35-mL aliquots of slurry were removed to 0.5-mL microcentrifuge tubes; 0.25 mL was removed from all supernatants for assay. The time course was started by adding 0.25 mL of buffer A containing 100 μM [^{3}H]TTX and with or without a saturating concentration of

N-acetylneuraminic acid (NeuNAc, 10 mM). Incubations were terminated by brief centrifugation at the times indicated in Figure 2B, and then aliquots of 0.2 mL were assayed. The rate of dissociation of sodium channels from the immunoresin (Figure 5A, inset) was measured by the same method.

Displacement from LFA–Sepharose with Sialic Acid. LFA–Sepharose was suspended in 2 volumes of medium A, and then aliquots of 300 μL were put in microcentrifuge tubes that already contained 18 pmol of detergent-solubilized Na channels in 100 μL of DP and 250 μL of NeuNAc, making the final concentrations indicated in the Figure 3. Incubations of 3 h were performed with end-over-end mixing in a cold room. After a brief spin in a microcentrifuge, 2 × 200-μL aliquots of supernatants were assayed for [^{3}H]TTX binding, and 2 × 20-μL aliquots were assayed for total protein. Resins were rinsed with 3 × 1.0 mL of buffer A. Then 550 μL of Na NeuNAc, 11.8 mM, was added, making a final concentration of 10.0 mM. After a 10-min incubation followed by a brief spin, aliquots were assayed as already described. Nonspecific counts subtracted from total counts were measured in replicate incubations with saturating unlabeled TTX. Competitions with samples in both series measured 53.3 cpm in the unbound supernatant and 33.7 cpm in the desorbed material. Specific binding was ~85% of maximum total binding.

Electrophoresis. Gradient SDS–polyacrylamide gels (4–15%) were prepared according to the method of Laemmli (1970). Proteins were heated at 60 °C for 10 min in 2% SDS (w/v), 5% 2-mercaptoethanol (v/v), 10% glycerol (v/v), and 0.0625 M Tris-HCl, pH 6.8. Protein was stained with Coomassie blue R-250 as described (Miller et al., 1983). Gels were scanned with an optical densitometer (Bio-Rad).

Preparation for Ion Stabilization Experiments. Starting with 90–120 g of crude electroplax membranes, sodium channels were purified through the DEAE column step as described above, except that after the resin was incubated with the membrane extract, the DEAE column was washed with 5 column volumes of buffer containing 25 mM Hepes–Tris, pH 7.2, 0.1% Lubrol-PX, 0.183 mg/mL phosphatidylcholine, 200 mM NaCl, and the protease inhibitors described above. TTX-binding activity was eluted with the same buffer containing 600 mM NaCl. The peak TTX-binding fractions were pooled and concentrated 7–9-fold with pressure (30 psi) over an Amicon XM-50 pressure-dialysis membrane and stored on ice for up to 24 h.

Ion Stabilization Assay. The pooled, concentrated DEAE desorbate (DPC) was desalted by the rapid gel filtration procedure of Levinson et al. (1979); 200 μL of DPC was applied to a 1.5-mL column of Sephadex G-50 preequilibrated at 20 °C with buffer A: 25 mM Hepes–Tris, pH 7.2, 0.1% Lubrol-PX, and 0.183 mg/mL PC. The column was centrifuged for 60 s at 1500 rpm (400*g*) in a Dynac II tabletop centrifuge (Clay Adams); 2.5 min after the centrifugation was started, 0.92 mL of column effluent was transferred to a tube preequilibrated at 20 °C containing 250 μL of buffer A supplemented with salt to give the final concentrations indicated in the legends to Figures 7–9. After 10 s, 180 μL of this mixture was transferred to a tube containing 70 μL of ice-cold "stop" solution: buffer A plus 1 M NaCl and either 15 μL of [^{3}H]TTX (3.94 μM, 145 dpm/pmol) plus 15 μL of H_2O (for measurement of total binding) or 15 μL of unlabeled TTX (100 μM, for measurement of nonspecific binding). The tube was immediately vortexed and plunged into ice. Controls demonstrated that no decrement in TTX binding occurred after this time. For experiments with zinc, 25 mM Tris was used in buffer A because precipitates formed with HEPES, and the stop solution contained 25 mM EDTA. The time of addition of the first 180-μL aliquot to the stop solution was taken as zero. The sample was incubated at 20 °C, and 180-μL aliquots were stopped as above at the times indicated in the figure legends. Five minutes after the last time point, 15 μL of [^{3}H]TTX was added to the competition tubes containing only unlabeled TTX and vortexed; 220 μL was withdrawn, and bound [^{3}H]TTX was measured as above.

The stability of the TTX-binding sites was represented in two ways. As a qualitative measure of relative potency of various stabilizing ions, the fractional decay of TTX binding activity in 18 min at 20 °C was used (Figure 7, triangles, and Figure 8). In Figures 7 (circles) and 9 the denaturation rate constant was used as a quantitative measure. This is the initial slope of $-\ln (B_t/B_0)$ vs t, where B_t is the concentration of TTX-binding sites remaining after t seconds.

Results

Lectin Affinity Resins. Eleven lectin–agarose resins were evaluated for uptake and release of [^{3}H]TTX-binding sites. These were tested with protein initially purified on DEAE-Sephadex as previously described (Rosenberg et al., 1984a). Optimal properties of such resins would be saturable uptake with sufficient resin, retention by the resin during a wash step, and rapid, specific desorption with high recovery of the adsorbed binding sites.

In this and a previous study (Moore et al., 1982), it was observed that nine of the lectin resins (listed under Materials and Methods) fail to take up the protein. Wheat germ agglutinin (WGA) was tested at a high lectin content (8 mg of protein/mL), as recommended by Furukawa et al. (1986). This resin yielded 90 pmol of [^{3}H]TTX-binding sites desorbed/mL of resin, a marked improvement. However, some DEAE preparations were not fully adsorbed even when small samples were applied, suggesting heterogeneity of carbohydrate chains. Because WGA does not bind polysialic acid (Bhavanandan & Katlic, 1979; Rutishauser et al., 1988), we tested LFA, a recently described lectin that specifically binds *N*-acetyl- and *N*-glycolylneuraminic acid residues (Miller & Cannon, 1984; Miller, 1987).

LFA–Sepharose was found to adsorb [^{3}H]TTX-binding sites tightly, and similar behavior was shown by WGA, as illustrated in Figure 1. Bound complexes were stable to washing, and the TTX-binding sites were eluted from both resins with the appropriate monosaccharides. When portions of the same DEAE fraction were fractionated in parallel with both lectin resins, LFA gave a markedly better purification, yielding material of higher specific activity. The difference was also apparent in densitometer scans of SDS–polyacrylamide gels stained with Coomassie blue (Figure 1C,D). In the most favorable case, the specific activity of the LFA fractions was about 2200 pmol/mg, and that of the WGA fraction was about 1600 pmol/mg compared to 4800 pmol/mg expected for pure, undenaturated protein. This was in agreement with the peptide compositions. The capacity of the LFA resin (5 mg of LFA/mL of resin) in 13 experiments exceeded 500 pmol of [^{3}H]TTX-binding sites/mL of resin.

Binding of Na Channels and Sialic Acid to LFA–Sepharose. We measured the rate of adsorption of sodium channels to LFA resin. As shown in Figure 2A, depletion of [^{3}H]TTX-binding sites from solution containing excess glycoprotein approached equilibrium in 60 min. The semilogarithmic plot of these data revealed a rapid uptake with a half-time of ~10 min, complete in about 45 min, and a slower rate of uptake that persisted past 90 min. The equilibrium concentration of

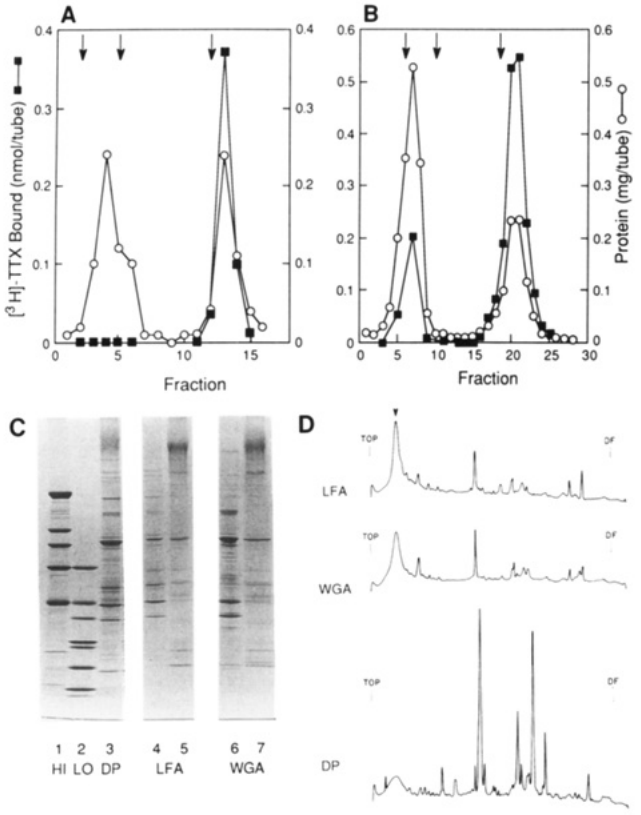

FIGURE 1: WGA and LFA lectin affinity chromatography. [^{3}H]-TTX-binding sites applied to columns of lectin resin remained tightly bound through a wash and then were eluted by the appropriate monosaccharide (WGA, *N*-acetylglucosamine, 100 mM; LFA, sodium *N*-acetylneuraminic acid, 10 mM). (A) To LFA–Sepharose 4B (4 mL of resin, 5 mg of LFA/mL of resin) was applied a DEAE fraction in which the specific activity was 784 pmol/mg of protein, containing 2150 pmol of [^{3}H]TTX-binding sites; 2005 pmol was eluted, of which 510 pmol was unbound and 1495 pmol was specifically desorbed. In the desorbate the specific activity was 2185 pmol/mg of protein. (B) To WGA–Sepharose 4B (6.0 mL of resin, 8 mg of WGA/mL of resin) was applied a DEAE fraction in which the specific activity was 650 pmol/mg of protein, containing 720 pmol total of [^{3}H]TTX-binding sites; 700 pmol of [^{3}H]TTX-binding sites was desorbed. In this the specific activity was 1640 pmol/mg of protein. (C) SDS–acrylamide gradient gels of peptides in LFA and WGA affinity resin fractions. The gels were stained with Coomassie blue (note that Na channel peptide is the band at ~280 kDa). (Lane 1) Molecular weight standards: myosin (205 000); β-galactosidase (116 000); phosphorylase *b* (97 400); bovine serum albumin (66 000); ovalbumin (45 000). (Lane 2) Molecular weight standards: bovine serum albumin (66 000); ovalbumin (45 000); glyceraldehyde-3-phosphate dehydrogenase (36 000); carbonic anhydrase (29 000); trypsinogen (24 000); trypsin inhibitor (20 100); and α-lactalbumin (14 200). (Lane 3) Initial DEAE fraction applied to lectin resins. (Lane 4) Unbound fraction from LFA. (Lane 5) Bound and desorbed fraction from LFA. (Lane 6) Unbound fraction from WGA. (Lane 7) Bound and desorpbed fraction from WGA. (D) Optical density scans of fractions. Arrowhead indicates 280-kDa channel peptide.

soluble [^{3}H]TTX-binding sites was about at the threshold of detection (1 nM) when <500 pmol of sample was applied per milliliter of resin. Any [^{3}H]TTX-binding sites eluted during extensive washes were undetectable, and the intrinsic off-rate could not readily be measured. Nevertheless, a rapid off-rate with a half-life of ca. 1 min was obtained upon addition of competing sialic acid (Figure 2B). Because release in the presence of *N*-acetylneuraminic acid (NeuNAc) was much more rapid than the rate of Na channel uptake, it seems likely that the lectin binds to multiple sites present on each sodium channel protein.

We examined the equilibrium displacement of the [^{3}H]-TTX-binding sites by varying concentrations of NeuNAc. In these experiments, with resin equilibrated with DEAE-fractionated sodium channels and NeuNAc, displacement of [^{3}H]TTX-binding protein from the resin shifted smoothly from 10% to 90% within a 100-fold range of the sialic acid (Figure 3). This corresponds to an effective affinity of about 0.45 mM sialic acid for a single class of binding sites. There was negligible offset between total protein uptake and the uptake of [^{3}H]TTX-binding sites. With successive steps of increasing concentrations of sialic acid and washes, portions of [^{3}H]-TTX-binding sites could be sequentially eluted from the resin, but no individual subfraction appeared to be more pure than any other (not shown). This again suggests that binding is most likely dominated by multiple, and somewhat heterogeneous, interactions of the protein's sialic acid substituents with the lectin matrix.

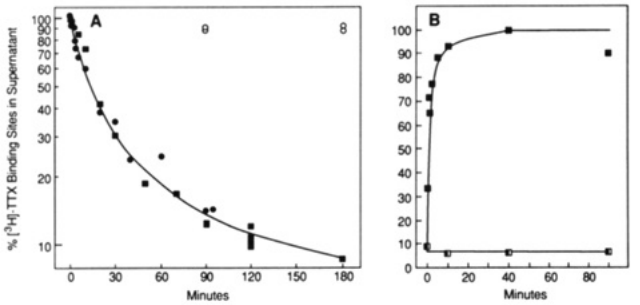

FIGURE 2: (A) Rate of adsorption of Na channels to LFA. [^{3}H]TTX binding was assayed in supernatants of incubations containing LFA–Sepharose 4B and a slight excess of DEAE-fractionated material. In one format (solid circles), separate incubations were performed and assayed, and in another format (solid squares), a column of lectin resin was perfused by recirculation. Controls: (open circles) Individual incubations to which saturating sialic acid was added. Initial slope of semilogarithmic plot gives a half-life of ~10 min, with an apparent slower component 180 min. (B) Rate of desorption of [^{3}H]TTX binding Na channels from LFA–Sepharose 4B resins when displaced by saturating *N*-acetylneuraminic acid (10 mM). Carbohydrate was applied to samples of the washed resin previously saturated with sodium channel, and then samples were centrifuged and supernatants were assayed. Solid symbols represent binding sites in supernatant after addition of ligand, half-solid symbols in the absence of ligand.

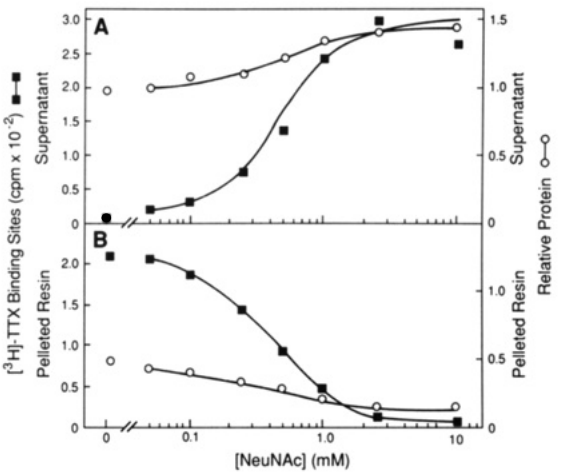

FIGURE 3: Displacement of [^{3}H]TTX binding sodium channels from LFA–Sepharose 4B at equilibrium. Individual samples were equilibrated with *N*-acetylneuraminic acid and a DEAE fraction, and then supernatants were assayed. (A) After initial incubation; (B) after rinsing resin and reapplying saturating (10 mM) NeuNAc. Uptake shifted smoothly from 10% to 90% within a 100-fold range of sialic acid, suggesting a single equilibrium constant for competition of 0.45 mM.

Purification of Anti-Polysialic Acid IgM. As recently reported (James & Agnew, 1988b), antibodies to bacterial α-(2→8)-linked polysialic acid bind almost exclusively to the

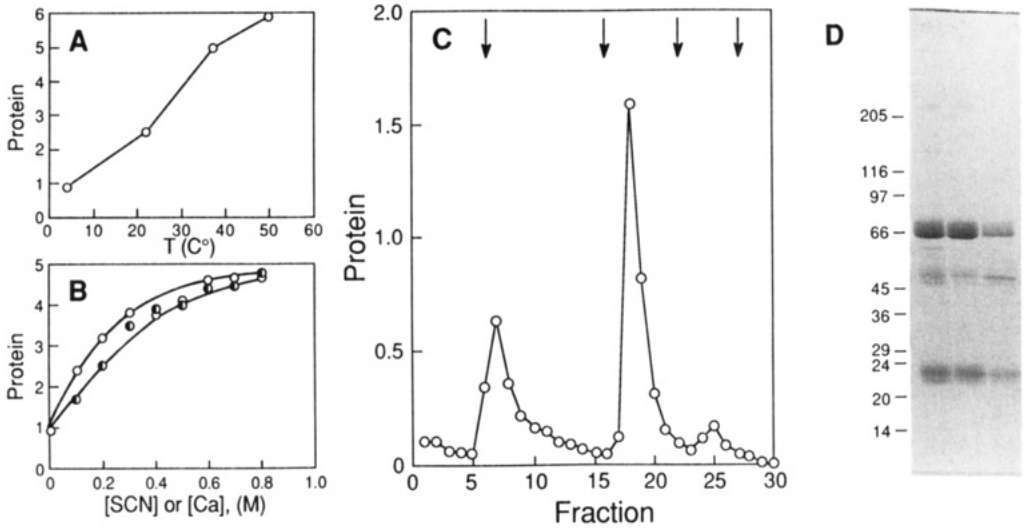

FIGURE 4: Specific antibody to α-(2→8)-polysialic acid was isolated by adsorption to an antigen-conjugated agarose resin and was eluted as follows. After extensive washing, purified antibody could be desorbed and affinity fractionated by gradual mild destabilization. (A) Temperature step gradients eluted increasing amounts of protein in individual incubations. (B) Antibody behaved similarly when the step gradients of the salts $CaCl_2$ or NaSCN were applied, also in individual incubations. (C) preparative elution from a chromatography column of antibody fractions with low-, medium-, or high-affinity binding, by desorption with a temperature step (4–22 °C) and two concentrations of NaSCN (0.5 and 3.0 M), applied respectively at arrows. Amounts of eluted protein were, 22 °C, 1.6 mg; 0.5 M SCN, 2.3 mg; 3.0 M SCN, 0.3 mg; final buffer wash, negligible. ([IgM] $\approx OD_{280}/1.2$; background from SCN subtracted). (D) SDS–acrylamide gradient gels of reduced peptides showed that all three subfractions contained purified IgM subunits.

[^{3}H]TTX binding Na channel in the electroplax. We tested an immunoaffinity strategy based on these findings. Antibodies to *N. meningitidis* capsular polysialic acid have been raised by others and, in particular, the immunochemical properties of a specific horse IgM are well established (Finne & Makëla, 1985; Jennings et al., 1985; Finne et al., 1987). Chains of 10 or more tandem sialosyl units are bound with progressively increasing affinity. We developed a procedure to purify highly specific antibody by selective adsorption to a synthetic antigen–resin carrying α-(2→8)-polysialic acid from the capsule of K1 strain of *E. coli* (colominic acid). To prepare the antigen for coupling to resin, the vicinal diol groups (on terminal sialosyl units) were first oxidized with sodium metaperiodate, and the aldehydes so formed were reacted with hydrazide-activated agarose. This coupling was chosen to be different from the linkage used to conjugate antigen to a carrier protein in the immunogen. Thus, the resin may be used to purify a rabbit antibody raised against colominic acid conjugated to BSA via a reduced Schiff base linkage (see below) because antibodies against the linkage will not be bound to the antigen resin. Elution of the antibody bound to this resin could be effected by mild destabilization. As shown in Figure 4, antibody was eluted in subfractions of varying affinity by increasing the buffer temperature or with increasing concentrations of NaSCN and $CaCl_2$. SDS–PAGE showed almost exclusively subunits of IgM in the subfractions eluted from the antigen–resin (Figure 4C,D). The antibody content of the horse serum recovered with the immunoresin was ~1.0 mg protein/mL, equal to the antibody that is immunoprecipitable with colominic acid from the same serum (Jennings et al., 1985). For these studies, antibody tightly bound at 4 °C was eluted with warm buffer (50 °C) and was then conjugated to CNBr-activated Sepharose resin, as described.

This can be used with anti-polysialic acid antibodies raised in rabbits. For example, rabbit antiserum was raised against a chemically modified polysialic acid (Jennings et al., 1986) that was first conjugated to protein as described by others (Jennings & Lugowski, 1981). After this antibody was purified against the same antigen resin it was successfully used with fixed *Staphylococcus aureus* cells to immunoprecipitate the eel Na channel (W. James and C. Ukomadu, unpublished data).

Immunoaffinity Chromatography. We have observed that colominic acid competes with the sodium channel for the antibody (James & Agnew, 1988b, 1989b). As shown in Figure 5A, inset, colominic acid causes very rapid desorption of [^{3}H]TTX-binding sites. At a saturating concentration of 10 mg/mL desorption was complete within 10 min. This is a concentration 40-fold above the K_i previously estimated by titration, and 5 mg/mL of carbohydrate may be routinely used for elution. To regenerate the resin, it was simply washed to remove colominic acid.

To test the IgM affinity resin, we began with a DEAE fraction, as in the case of LFA and WGA resins. In the experiment illustrated in Figure 5A, the DEAE fraction was equilibrated with IgM resin as a slurry for ~1 h. The resin was poured into a column and was washed with ~1.5 column volumes of buffer. (All steps were in the absence of stabilizing TTX.) The column was eluted with colominic acid. From this resin (containing ~87 mg of IgM), 340 pmol was obtained in the 1.05-mL peak fraction at a specific activity of 2664 pmol/mg. This may be compared to the theoretical maximum of 4800 pmol/mg, calculated by assuming one [^{3}H]TTX-binding site per 208 000 Da of peptide chain (Table I). When run on SDS gels (Figure 5B,C), immunopurified material appeared as a highly purified peptide of ~285 000 Da. This material was routinely of higher specific activity and simpler peptide composition than LFA-purified material (Figure 6), and serial fractionation by DEAE, LFA, and IgM resins offered no improvement (Figure 6).

As serial affinity chromatography was tried without further improvement in the specific activity, as material purified by DEAE and IgM chromatography was essentially pure, and as recovery for the second step was here ~85% of the activity applied to the column, we concluded that the lower than maximum specific activity likely results from denaturation on DEAE. Nevertheless, the specific activity and activity recovered are higher than had previously been attained with DEAE and one or two cycles of molecular sieving, even in the presence of stabilizing [^{3}H]TTX (Table I).

Table I: Purification of Eel Electroplax Sodium Channels

	TTX binding			
	specific activity[a]		recovery[b]	
	pmol/mg	stoichiometry	pmol	% initial
Lubrol-PX, phosphatidylcholine				
IgM–Sepharose[c]	3196	0.67	420	
IgM–Sepharose (n = 4)	2986 ± 49	0.62	1501 ± 72	92 ± 5
DEAE-Sephadex				
IgM–Sepharose[c]	2664	0.56	338	32
LFA–Sepharose[c]	2187	0.46	1496	40
WGA–Sepharose[c]	1640	0.34	698	
WGA–Sepharose[d]	1420	0.30	104	9
DEAE-Sephadex[e]	229			61
Sepharose 6B + TTX[e]	1698	0.35	1770	41
Sepharose 6B repeated[e]	2150	0.45	1133	26
CHAPS, phosphatidylcholine, asolectin				
DEAE-Sephadex[f]				76
Sepharose 6B + TTX[f]				59
Sepharose 6B repeated[f] (n = 12)	2387 ± 140	0.50		38 ± 2

[a] In usable peak fractions; compared with maximum of 4800 assuming one site per 208 000 daltons of polypeptide chain. [b] Total in fraction. [c] Reported here. [d] Norman et al. (1983). [e] Agnew et al. (1978). [f] Duch and Levinson (1987).

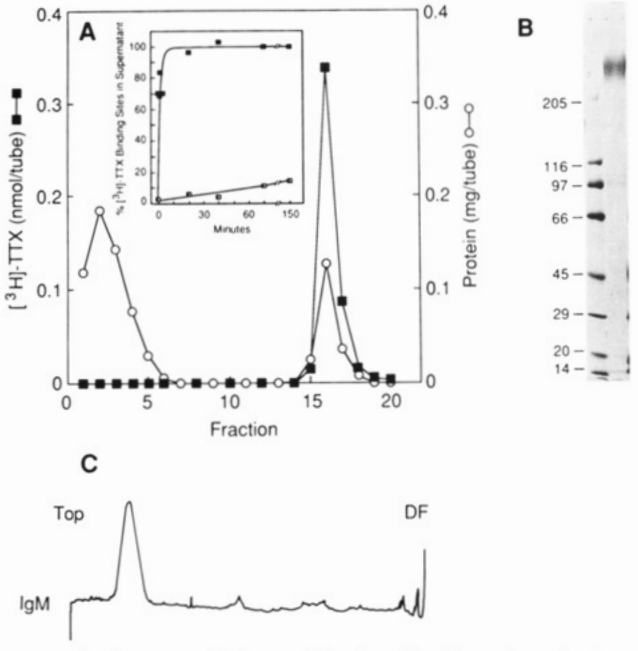

FIGURE 5: Immunoaffinity purification of sodium channels from DEAE-fractionated material with IgM–Sepharose 4B. (A) The binding was performed in a slurry (87 mg of IgM on 10.6 mL of resin; 580 pmol of DEAE fraction applied, originally 431 pmol/mg) for 90 min, and then the resin was poured into a column (0.7 × 24 cm) that was washed with 1.5 bed volumes, after which the bound channels were eluted with colominic acid, 5 mg/mL. Fractions of 1.05 mL were collected and assayed as described under Materials and Methods. (Inset) α-(2→8)-polysialic acid from *E. coli* (colominic acid, 10 mg/mL, ca. 0.05 mM) was applied to IgM immunoresin samples preincubated with sodium channels. Desorption was immediate and complete by ~10 min. (B) SDS–acrylamide gel of fraction 15, stained with Coomassie blue. The immunopurified Na channels appeared as the polydisperse band at ~285 kDa. (C) Optical density scan of Coomassie-stained gel demonstrates that the protein was purified essentially to homogeneity.

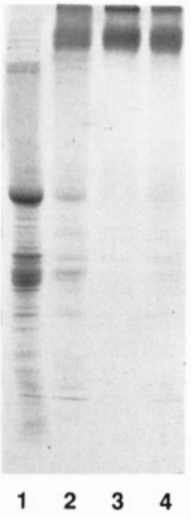

FIGURE 6: Sodium channel polypeptide fractions obtained from LFA, IgM, or both sequentially. Column chromatographies were performed as described (Materials and Methods). (Lane 1) Lubrol-PX extract of membranes; (lane 2) peptide composition after fractionation on DEAE and LFA; (lane 3) peptide composition after fractionation on DEAE, LFA, and IgM; (lane 4) peptide composition after DEAE and IgM chromatographies.

To avoid denaturation during ion exchange, we tested the feasibility of single-step purification from the initial detergent extract. When 1% Lubrol-PX extract was appied directly to LFA resin, results were not satisfactory. However, we found that a 1% Lubrol-PX extract of membranes could be used with the IgM resin, exactly as for the DEAE-purified fractions. After washing with standard buffer, colominic acid was used to elute protein of specific activity ~3200 pmol/mg, approximately 67% of the theoretical maximum. Recoveries here averaged 92%. The maximum specific activities were achieved when membranes were first fractionated by differential sedimentation, as described under Materials and Methods; however, specific activities averaging 3000 pmol/mg were obtained with the previously described membranes (Agnew et al., 1978). On SDS–polyacrylamide gel electrophoresis, this material was essentially indistinguishable from that observed with the two-step procedure. Thus, a swifter one-step fractionation yielded the highest specific activity yet reported for any sodium channel preparation. These different experiments are compared in Table I.

Ionic Stabilization of TTX Binding Activity. Incidental to these studies we examined the effects of ionic strength on the stability of the TTX-binding site. Studies with solubilized rat brain (Catterall et al., 1979) sodium channels have shown that, in addition to a minimal ratio of phospholipid to detergent, calcium ion is required for STX-binding site stability. Calcium ion was also included during the purification of the rat muscle sodium channel to stabilize saxitoxin binding [cf. Barchi (1983)]. The electroplax channel has been found to be stable without added Ca^{2+} under the purification conditions previ-

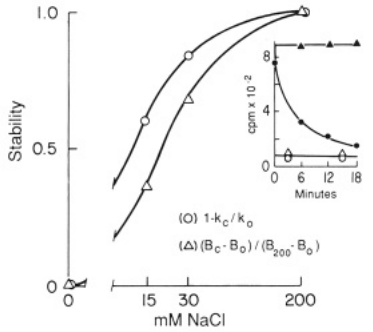

FIGURE 7: NaCl stabilizes the TTX-binding site against thermal denaturation. (Inset) Time course of TTX binding during incubation at 20 °C in buffer A (circles) and in buffer A plus 200 mM NaCl (triangles); (solid symbols) total [^{3}H]TTX binding; (open symbols) nonspecific binding. (Stability curve) Two measures of stability are shown here. (1) The triangles represent simply the fraction of binding sites preserved (B_c–B_0) at the indicated NaCl concentration (c), compared to those preserved in 200 mM NaCl (B_{200}–B_0); B_0 is the small amount of binding activity remaining after 18 min without added NaCl (see inset). This measure of stability is useful for comparing the relative stabilizing potency of different ionic solutions and was used for Figure 8. (2) The circles represent values derived from actual decay rate constants, measured as the magnitude of the slope of the linear, semilog plot of the decay time course. k_0 is the decay rate constant in buffer A alone; k_c is that for buffer A plus NaCl at the indicated concentration (c). $(1 - k_c/k_0) = 1$ means no decay was detectable in 18 min. The midpoint of the stabilization curve ($1 - k_c/k_0 = 0.5$) gives the salt concentration that reduces the decay rate by half.

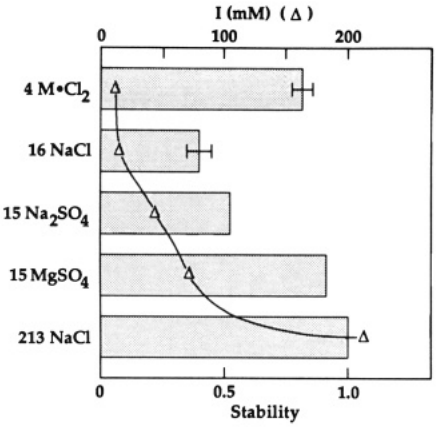

FIGURE 8: Dependence of stability on ionic composition. Stability measures the survival of [^{3}H]TTX-binding sites after 18 min at 20 °C, as in Figure 7(1). Stability is the fraction of TTX-binding sites preserved in buffer A plus the indicated salt compared to buffer A alone. A stability value of 1 indicates no loss of binding activity; a stability value of 0 indicates decay equal to that in buffer A without added salt. All concentrations are in millimolar. The contribution of the salt to the ionic strength (I) is given in each case by Δ, with $I = (0.5)(m_+z_+^2 + m_-z_-^2)$, where m_+ and z_+ are the concentration and valence, respectively, of the cationic component of the salt, and m_- and z_- are the corresponding values for the anion. Each value is the average of two determinations, except for 16 NaCl, which is the average of duplicate assays from four separate experiments, and 4 MCl_2, which is the average of four sets of duplicates, one for each of the chloride salts of the divalent cations (M) Mg^{2+}, Mn^{2+}, Ba^{2+}, and Ca^{2+}. Error bars give standard deviations.

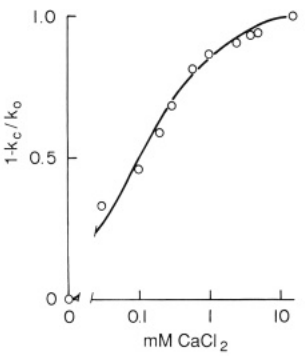

FIGURE 9: Effect of calcium ion on stability. Stability is derived from denaturation rate constants as described in Figure 7(2). k_0 is the decay rate constant in buffer A alone; k_c is the rate constant in buffer A plus the indicated $CaCl_2$ concentration.

ously reported (Agnew et al., 1978; Agnew & Raftery, 1979). We found in these studies, however, that reducing the ionic strength of a solubilized preparation (initially in 50 mM sodium phosphate), by dialysis or gel filtration, caused a rapid loss of TTX binding activity that was not restored by readdition of salt (M. Emerick, unpublished data). The loss of activity was complete within 4 h at 0 °C, and the denaturation rate was enhanced at elevated temperature. The experiments described below were performed at 20 °C without stabilizing TTX, where substantial denaturation could be obtained within 20 min (see Figure 7, inset).

Figure 7 shows that when all inorganic ions were replaced by 50 mM Hepes–Tris buffer in a rapid gel filtration step, binding activity decayed rapidly with approximately first-order kinetics. Supplementation of the incubation buffer with increasing concentrations of NaCl lowered the denaturation rate, with a midpoint for stabilization of about 10 mM. In the experiments illustrated in Figure 8 we used this experimental format to test whether stabilization was ion selective or a nonspecific effect of ionic strength. It is clear from Figure 8 that stability was not correlated with ionic strength per se, but is probably a more specific effect of the ions themselves. It is also evident from this figure that neither of the anions tested (Cl^- and SO_4^{2-}) can account for the observed dependence of stability on salt concentration. This indicates that the stabilizing effects of a salt are attributable to the cation. We tested the chloride salts of five alkali metals (Li^+, Na^+, K^+, Cs^+, and Rb^+) for evidence of selectivity, but no significant differences were found (not shown). The data of Figure 8, however, indicate that divalent cations are more potent stabilizers than are monovalent cations. This is borne out in Figure 9, which shows a stability curve for $CaCl_2$. The midpoint for stabilization by $CaCl_2$ was 100 μM, or 100-fold lower than that for NaCl. As with the monovalent cations, several divalent cations were tested for evidence of selectivity, and no differences were found between Mg^{2+}, Ca^{2+}, Ba^{2+}, and Mn^{2+}. Zn^{2+} was found to be destabilizing, however. Denaturation was more rapid in the presence of Zn^{2+} than in the absence of any added salt (data not shown). This effect was partially overcome by the addition of 200 mM NaCl and was not the result of simple competition between Zn^{2+} and TTX. Thus, divalent cations, with the notable exception of zinc, were effective at much lower concentrations than monovalent cations at stabilizing the solubilized TTX binding site.

Discussion

The carbohydrate substituents of the electroplax sodium channel are a prominent structural feature. The recently discovered polysialic acid chains (James & Agnew, 1987, 1988b, 1989b) appear to contribute to the reported electrophoretic microheterogeneity (Miller et al., 1983) and may explain the avid binding of the protein to anion-exchange resins and the unusually large Stokes radius measured by gel filtration (Agnew et al., 1978, 1986). They now provide the basis for the affinity purification schemes described here.

Affinity chromatography is an attractive separation method because it is mild and rapid and may be performed on large and small scales. Perhaps because binding to the affinity resins

involves only the branches of carbohydrates and because the separation is very fast, the [^{3}H]TTX-binding-site recovery is reproducibly as high as 92% (Table I). Because of the selectivity of the IgM resin, the previous DEAE and gel filtration steps, as well as pressure concentration, can all be eliminated. This avoids the need for stabilizing TTX (Agnew et al., 1978; Duch & Levinson, 1987) and the inevitable loss of activity occurring with the DEAE step (Agnew et al., 1978; Miller et al. 1983; Duch & Levinson, 1987). In previous reconstitution studies, stabilizing TTX had to be removed (Duch & Levinson, 1987), or losses incurred during gel filtration in its absence had to be tolerated (Rosenberg et al., 1984a,b; Tomiko et al., 1986; Agnew et al., 1986). The new method for membrane fractionation takes less time than that used earlier with only slightly lower yield (Agnew et al., 1978; cf. Table I).

Affinity purification from LFA or IgM resins yields protein that is active in reconstituted vesicles (Cooper & Agnew, 1989; Cooper et al., 1987; Agnew et al., 1988), excised patches from expanded liposomes (Correa & Agnew, 1988; Correa et al., 1989; Agnew et al., 1988), and planar bilayers (Schenkel et al., 1989). The affinity resins permit control of reconstitution conditions. The protein can be eluted at concentrations exceeding 1 mg/mL. For a constant bed volume, the concentration of protein eluted with a step gradient of sialic or polysialic acid is approximately proportional to the height of the column. Long, thin columns yield protein at higher concentrations, without concomitant concentration of stabilizing detergent/lipid micelles as does occur with pressure ultrafiltration. Thus, the protein to lipid ratio in reconstituted vesicles can be varied. Also, both the detergent and the lipid composition of the sample can be changed while the protein is bound to the resin, before reconstitution. Vesicles produced with high protein to lipid ratio have markedly improved success in patch-clamp recording from reconstituted liposomes (Correa & Agnew, 1988; Correa et al., 1989, and unpublished results).

The anti-polysialic acid IgM resin takes advantage of antibodies raised against the bacterial antigen, which are typically of low affinity (Finne et al., 1987). Immunochemical studies suggest that the actual epitope of the polysialic acid is a conformationally determined or "topographical" type, consisting of a decamer in which the flanking residues stabilize the conformation of the central antibody binding residues (Michon et al., 1987). Thus, the avidity increases with chain length. With pure carbohydrate, lower temperatures appear to stabilize the immunoreactive conformation (Mandrell & Zollinger, 1982), temperatures that favor sodium channel stability. The IgM resin binds the sodium channel at concentrations more than 3 orders of magnitude lower than the concentration of bacterial antigen required for 50% competitive displacement (James & Agnew, 1988b, 1989b). However, the sodium channel is readily displaced by bacterial polysialic acid. In contrast, monoclonal antibodies raised against eel or rat skeletal muscle sodium channels exhibited avidities so high that nondenaturing elution conditions were not found (Nakayama et al., 1982; Casadei et al., 1986). An appealing explanation is that the stable immunoresin complex involves multiple, low-affinity interactions with polysialic acid substituents on the Na channel protein. The binding properties of LFA and WGA resins also suggest multiple sites of lectin–sodium channel interaction.

The one-step immunofractionation of protein solubilized from the membrane preparation described here yields material of higher specific activity than reported for other methods (Table I; also cf. Hartshorne and Catterall (1984), Elmer et al. (1985), Kraner et al. (1985), Barchi (1983), and Lombet & Lazdunski (1984)]. Colominic acid can be effectively reduced by dialysis (e.g., allowing for repeated immune-affinity adsorption; W. James, unpublished data) or a small-scale gel filtration if necessary. Large-scale immunoresin preparation is feasible (Table I), with antibodies from horse serum (Jennings et al., 1985), monoclonal hybridoma lines (Mandrell & Zollinger, 1982; Frosch et al., 1985), or rabbits immunized with modified and protein-conjugated polysialic acid (Jennings et al., 1986; W. James and C. Ukomadu, unpublished data; see Methods).

The IgM resin described here may not permit purification of mammalian brain sodium channels. We failed to demonstrate binding to brain protein by immunoblot or in affinity uptake experiments. Although it has not been reported whether α-(2→8)-polysialic acid specific endoneuraminidase cleaves carbohydrate from the brain protein, the synthesizing enzyme for this carbohydrate does not occur in measurable levels in adult brain (McCoy et al., 1985). WGA resins are used to purify mammalian brain and muscle sodium channels (Hartshorne & Catterall, 1981, 1984; Barchi et al., 1980; Kraner et al., 1985), and we have found that LFA is superior to WGA in preparing the rat brain proteins (W. James, unpublished observations).

The stabilization experiments show that the solubilized protein is denatured quickly at low ionic strength. Stabilization by salts is not a general consequence of ionic strength and is due primarily to the cationic components. In circumstances where relatively low ionic strength is desired, divalent cations may be substituted for monovalent cations at approximately 100-fold lower concentrations. In addition, substitutions may be made among cations of the same valence without affecting stability. The stabilization by divalent cations of the solubilized electroplax sodium channel is consistent with the effect of calcium observed in rat brain and muscle preparations and occurs over the same concentration range. Our results differ in that divalent cations can be replaced by monovalent cations (at a higher concentration). This stabilization was not observed previously in electroplax preparations because 0.2 M KCl was added to the solubilized membrane extract shortly after preparation. The substitution of other ions for calcium has not been reported for the rat brain preparations, although in more recent work on the rat muscle channel magnesium (500 μM) has been used to replace calcium (Kraner et al., 1985).

The effects of Zn^{2+}, in particular, merit further attention. Although the destabilization by Zn^{2+} could be due to formation of complexes with a sulfhydryl necessary for TTX binding or conformational stability, neither reducing agents (dithiothreitol, 2-mercaptoethanol) nor alkylating agents such as iodoacetamide, iodoacetic acid, or *N*-ethylmaleimide alter the stability of TTX binding (W. Agnew, unpublished data).

Acknowledgments

We thank J. B. Robbins for providing immune horse serum against α-2,8-polysialic acid and Hana Rudnick for her expert technical assistance.

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