Biosynthesis of Electroplax Sodium Channels in *Electrophorus* Electrocytes and *Xenopus* Oocytes†

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ABSTRACT: We have synthesized the eel electroplax sodium channel core polypeptide in both a cell-free and a frog oocyte system and report it does not possess the unusual electrophoretic properties of the mature, native sodium channel polypeptide isolated from electroplax membranes. On sodium dodecyl sulfate–polyacrylamide gel electrophoresis, the mature channel polypeptide exhibits both a diffuse banding pattern (microheterogeneity) and an extremely high electrophoretic free mobility. In contrast, the core polypeptide synthesized in vitro or in vivo migrates as a sharp band with a near-normal electrophoretic free mobility (M_r 230 000). The microheterogeneity of the mature peptide has been inferred to result from varying degrees of glycosylation of the channel polypeptide [Miller, J. A., Agnew, W. S., & Levinson, S. R. (1983) *Biochemistry 22*, 462–470]. We present evidence here that the anomalously high electrophoretic free mobility is due to the binding of large amounts of sodium dodecyl sulfate to posttranslationally modified domains on the protein. In addition, we have followed the posttranslational processing of eel sodium channels in both the eel electrocyte and the frog oocyte. Using lectin binding and Ferguson analysis, we found that the channel was processed relatively rapidly to an intermediate form in the Golgi apparatus that apparently contained fewer carbohydrate and hydrophobic domains than the mature channel. The further addition of carbohydrate and hydrophobic domains, which are required before the channel acquires its characteristic physicochemical properties, proceeded relatively slowly in the electrocyte and appeared not to have occurred to the majority of intermediately processed channels in the frog oocyte.

The voltage-dependent sodium channel plays a central role in the initiation and propagation of the action potential in most electrically excitable tissue. Electrophysiological studies have given precise information on the sodium channel's voltage dependence, ion selectivity, and kinetics of gating functions, as well as the magnitude of ion currents which flow through a single open channel (Hodgkin et al., 1952; Aidley, 1978). Although the sodium channel has been extensively studied using biophysical and pharmacological techniques, it has only been in recent years that attention has focused on the isolation and biochemical characterization of this important membrane protein (Levinson, 1981; Barchi, 1982; Agnew, 1984; Catterall, 1984). The ultimate goal of this biochemical approach is to correlate the wealth of functional information available on the sodium channel with the structure of the molecule to gain insights into the operation of the channel on a molecular level.

The biochemical isolation of the sodium channel protein has been achieved by using the binding of tritiated neurotoxins, tetrodotoxin (TTX)[1] or saxitoxin (STX), as assays for the channel in detergent solutions (Agnew et al., 1978). The TTX binding component from electroplax membranes of *Electrophorus electricus* has been shown to be a large, single polypeptide with an apparent molecular mass of approximately 260–290 kilodaltons (Miller et al., 1983). This channel protein has also been found to be heavily glycosylated, consisting of about 30% carbohydrate of which 40% is sialic acid. Several reconstitution studies have shown that this toxin binding polypeptide isolated from the electroplax is capable of demonstrating many of the characterisitics of normal channel function (Rosenberg et al., 1984; Duch & Levinson, 1985). Most notably, the highly purified, large polypeptide exhibits a veratridine-stimulated, TTX-blockable ^{22}Na flux when reinserted into lipid vesicles, suggesting it is a functional sodium channel. This highly purified polypeptide has also been incorporated into planar bilayers and has shown many of the functional attributes of a sodium channel (Levinson et al., 1986b).

The focus of this paper is 2-fold. We report the cell-free and cellular biosynthesis of the eel electroplax sodium channel polypeptide, and we contrast some physicochemical properties of this unmodified core protein with those of an apparent intermediate form in the frog oocyte as well as with the mature, native molecule from electroplax membranes. These studies indicate that the hydrophobicity of the mature channel is due to a posttranslational modification. We also have investigated the biosynthetic pathway that the eel sodium channel takes in both the eel electrocyte and the frog oocyte. Since the frog oocyte has become a model system to test for the electrophysiological expression of foreign ion channels and their altered mRNAs (Mishina et al., 1985; Noda et al., 1986b), the investigation of the biochemical expression and posttranslational processing of the sodium channel in the oocyte should determine whether it is processed similarly to the parent electroplax tissue. We present evidence that the eel sodium channel has a very complex biosynthetic history and that the assignment of molecular weights to intermediately processed

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[1] Abbreviations: TTX, tetrodotoxin; STX, saxitoxin; PAGE, polyacrylamide gel electrophoresis; $NaDodSO_4$, sodium dodecyl sulfate; CMC, critical micelle concentration; BSA, bovine serum albumin; WGA, wheat germ agglutinin; M_r, apparent molecular weight; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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channels must be made with care. We also found that the oocyte apparently did not completely posttranslationally process the majority of eel sodium channels to the mature form, thus providing an explanation for the apparent inefficiency of functional expression of these channels in the oocyte (Agnew, 1986).

Materials and Methods

Materials. Electric eels were obtained from World Wide Scientific Products. Animals were killed by hypothermia upon arrival and the electric organs removed and frozen at −80 °C. Adult *Xenopus laevis* were obtained from the South African Snake Farm. The rabbit reticulocyte lysate system and [^{35}S]methionine (>1100 Ci/mmol) were obtained from New England Nuclear. Na^{125}I and sodium dodecyl [^{35}S]sulfate were purchased from Amersham, and the ^{14}C-labeled protein standards and human placenta RNase inhibitor were from Bethesda Research Labs. Protein A–Sepharose CL-4B and the various protease inhibitors were from Sigma Chemical or Boehringer, Mannheim. Wheat germ agglutinin–Sepharose 4B was obtained from Pharmacia.

Purification of the Eel Sodium Channel and Antibody Production. The methods for the isolation of the sodium channel from electroplax tissue of the electric eel have been extensively described (Miller et al., 1983). For both immunizations and iodination, the highly purified glycopeptide obtained by $NaDodSO_4$ gel filtration on Sepharose 4B was used. Immunization of rabbits, purification of antibodies, measurement of antichannel titers, and ^{125}I iodination of the channel polypeptide were performed as previously described (Ellisman & Levinson, 1982).

$NaDodSO_4$ Binding. The binding of [^{35}S]$NaDodSO_4$ to the mature sodium channel polypeptide was determined by equilibrium dialysis at two different ionic compositions. This allowed us to determine the binding of the detergent at two different critical micelle concentrations. Low ionic strength was with 25 mM sodium phosphate, and high ionic strength was with 25 mM sodium phosphate + 200 mM NaCl, both at pH 6.8. Spectrapore Semi-Micro 4-mm tubing was prepared as recommended by the manufacturer and then stored in 0.1% $NaDodSO_4$/25 mM sodium phosphate buffer. Before use, the tubing was rinsed in distilled water and excess fluid removed.

Channel glycopeptide prepared by $NaDodSO_4$–Sepharose 4B chromatography was extensively dialyzed for 3 days against frequent changes of 25 mM sodium phosphate buffer, pH 6.8, to remove unlabeled $NaDodSO_4$. That this procedure does remove nearly all $NaDodSO_4$ was shown in control experiments in which [^{35}S]$NaDodSO_4$ was complexed to native protein and then dialyzed as above; at the end of 3 days, less than 0.05 g of $NaDodSO_4$/g of protein remained bound.

To measure the binding of $NaDodSO_4$, 10–20 μg of channel polypeptide in 50 μL of dialysis buffer was aliquotted into the dialysis tubing and placed in a 0.5-mL polyethylene microfuge tube containing 0.2 mL of dialysate. At the start of dialysis, varying concentrations of [^{35}S]$NaDodSO_4$ were placed either outside or inside the dialysis tubing, or at equal starting concentrations at both sides, and the tubes were then shaken at room temperature for 2–4 days to allow for complete equilibration. The time constant for equilibration in this system is about 6 h (K. McKennett and S. R. Levinson, unpublished results), and the final results were not affected by the initial placement of the radiolabeled detergent. After dialysis was complete, the volume and protein concentration of the bag contents were determined to correct for bag swelling or shrinking (mainly due to osmotic effects of $NaDodSO_4$), and aliquots of the bag contents and dialysate were counted by scintillation methods to determine [^{35}S]$NaDodSO_4$ concentrations and amount bound to protein. Binding of [^{35}S]-$NaDodSO_4$ was also determined for bovine serum albumin (Sigma grade V).

Extraction of Electroplax RNA. Total cellular RNA was extracted from eel electroplax tissue by the guanidinium thiocyanate method (Chirgwin et al., 1979). Poly(A) mRNA was isolated by affinity chromatography on oligo(dT)–cellulose (Avid & Leder, 1972). The isolated RNA was dissolved in sterile water and stored in aliquots at −80 °C.

Cell-Free Protein Synthesis and Immunoprecipitation. Total electroplax RNA was translated in a nuclease-treated rabbit reticulocyte lysate system (Pelham & Jackson, 1976). The optimal concentrations of RNA, K^+, and Mg^{2+} added to the system were determined per the manufacturer's directions. The system was supplemented with human placental RNase inhibitor (16 units/mL) and a cocktail of protease inhibitors (pepstatin A, chymostatin, antipain, and leupeptin were all at a final concentration of 0.1 μg/mL, while trasylol was added to 10 units/mL) (Walter et al., 1981). The addition of electroplax RNA to the translation mixture stimulated protein synthesis 20–30-fold above control. This was estimated by spotting an aliquot of the mixture on Whatman 3 paper and boiling in 10% trichloroacetic acid. Radioactivity was determined by liquid scintillation counting. In a typical experiment, approximately 1.0 A_{260} unit of RNA was translated in 100 μL of the reaction mixture containing 0.250 mCi of [^{35}S]methionine for 2–3 h at 31 °C. Translation was terminated by adding $NaDodSO_4$ to 4% and heating to 100 °C. An equal volume of H_2O was added followed by 4 volumes of immunoprecipitation buffer (190 mM NaCl, 6 mM EDTA, 50 mM Tris-HCl, pH 7.5, 2.5% Triton X-100, and 100 units of trasylol/mL). The translation mixture was precleared with preimmune rabbit sera and protein A–Sepharose beads for 4 h at 4 °C, followed by incubation with the specific antichannel rabbit antisera for 19 h at 4 °C and immunoprecipitation with protein A–Sepharose for 3 h. The immunoprecipitates were washed 5 times (1 mL per wash) with immunowash buffer (150 mM NaCl, 6 mM EDTA, 50 mM Tris-HCl, pH 7.5, 0.1% Triton X-100, 0.02% $NaDodSO_4$, and 100 units of trasylol/mL) and eluted as described (Anderson & Blobel, 1983).

Oocyte Protein Synthesis. Oocytes of *Xenopus laevis* were obtained and maintained as previously described (Colman, 1984). Oocytes were microinjected with 30–45-nL aliquots of electroplax mRNA (1 mg/mL) or total RNA (8 mg/mL) and incubated in incubation media with 2 mCi/mL [^{35}S]-methionine for different periods of time at 21–23 °C (Colman, 1984). Chase experiments used 10 mM methionine. At the end of the incubation period, any unhealthy oocytes were removed, and the remaining oocytes were homogenized in 25 μL per oocyte of solubilization buffer (4% $NaDodSO_4$, 10 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, and 50 mM Tris, pH 7.5) and heated to 100 °C. This was followed by the addition of an equal volume of water and 4 volumes of immunoprecipitation buffer and filtration through a sterile 0.45-μm nylon filter to remove the insoluble yolk and pigment particles. The immunoprecipitation of eel sodium channels was then carried out as described above. Noninjected or sham-injected oocytes never produced radioactive polypeptides that were precipitated by sodium channel antisera. For lectin affinity chromatography of glycosylated sodium channels, the following procedure was used: After the immunoprecipitate was washed, the bound sodium channel was eluted with three successive applications of 2% $NaDodSO_4$ (50 μL). Next, 1.3

mL of lectin buffer (250 mM KCl, 10 mM $CaCl_2$, 2.5% Triton, and 20 mM Hepes, pH 7.4) was added, followed by 50 μL of wheat germ agglutinin (WGA)–Sepharose 4B (2 mg of WGA protein/mL of gel matrix) in the presence or absence 100 mM *N*-acetylglucosamine. This solution was shaken for 5 h at room temperature, and the beads were then washed with 1 mL (5×) of lectin wash buffer (same as lectin buffer, except 150 mM KCl and 0.2% Triton) in the presence or absence of 100 mM *N*-acetylglucosamine. Any bound molecules were eluted with $NaDodSO_4$ sample buffer.

Electrocyte Isolation and Incubation. Electrocytes were isolated from the Organ of Sachs as described (Shoffeniels & Nachmansohn, 1957). Electrocytes were incubated in electrocyte buffer (170 mM NaCl, 5 mM KCl, 3 mM $CaCl_2$, 1.5 mM $MgCl_2$, 1.5 mM $NaHPO_4$, 15 mM Hepes, pH 7.4, 10 mM glucose, 1 mM pyruvate, and 200 μg/mL gentamicin) and 1 mCi/mL [^{35}S]methionine for different periods of time at room temperature. Chase experiments used 10 mM methionine. At the end of the incubation, the electrocytes were homogenized in solubilization buffer, heated to 100 °C, and processed as described above.

Gel Electrophoresis. $NaDodSO_4$–polyacrylamide gel electrophoresis was performed according to Laemmli (1970). Alkylation of the sodium channel core polypeptide or the native channel with iodoacetamide did not alter their mobilities on $NaDodSO_4$ gels. The radioactive proteins were visualized by fluorography as described (Bonner & Lasker, 1974).

Results

Relationship between Electrophoretic Free Mobility and $NaDodSO_4$ Binding. Ferguson analysis, in which the porosity of a separating polyacrylamide gel is varied, is used to estimate the electrophoretic mobility of a protein in free solution (Ferguson & Wallace, 1961). This analysis is an important test of the validity of $NaDodSO_4$–PAGE for molecular weight determinations of a given protein, because it essentially determines the electrophoretic driving force acting on the peptide during SDS–PAGE. In addition, important information about the physicochemical nature of a protein may be derived from its electrophoretic behavior in this analysis, particularly if such behavior is anomalous (Pitt-Rivers & Impiombato, 1968; Reynolds & Tanford, 1970a,b; Grefrath & Reynolds, 1974; Leach et al., 1980; Miller et al., 1983). To obtain free solution mobilities by this method, the *relative* mobilities measured for varying gel porosities are extrapolated to estimate the mobility of the protein in the absence of a gel matrix (e.g., see Figure 3).

Numerous studies [e.g., see Pitt-Rivers and Impiombat (1968) and Reynolds and Tanford (1970a,b)] show that most sulfhydryl-reduced, $NaDodSO_4$-denatured polypeptides form rod-shaped structures and bind a constant amount of negatively charged $NaDodSO_4$ per unit mass. The amount of detergent bound, about 1.0–1.4 g of $NaDodSO_4$/g of protein, represents a much greater amount of negative charge than that conferred by charged amino acid side groups or other moieties on the protein. Thus, different sized $NaDodSO_4$-denatured polypeptides usually experience the same electrophoretic driving force on $NaDodSO_4$–PAGE, and separation proceeds only on the basis of a molecular sieving of the different rod lengths on the porous acrylamide support. Naturally, such "well-behaved" peptides show very similar electrophoretic free mobilities on Ferguson plots (0% T in Figure 3).

However, anomalous separation behavior can result if the polypeptide fails to unfold into an extended rod in $NaDodSO_4$ or binds more or less $NaDodSO_4$ than expected. A polypeptide that binds unusually high amounts of $NaDodSO_4$ would have a high electrophoretic *free mobility*, since its charge to mass ratio, and hence its driving force, would be greater than that for standard proteins. Thus, the amount of $NaDodSO_4$ bound to the polypeptide and its electrophoretic behavior are intimately related. One consequence of such an altered driving force is that size determinations obtained from comparisons with the migration of standard protein on $NaDodSO_4$–PAGE are invalid, since the frictional component of the gel behavior tends to be offset by the increased electrophoretic mobility.

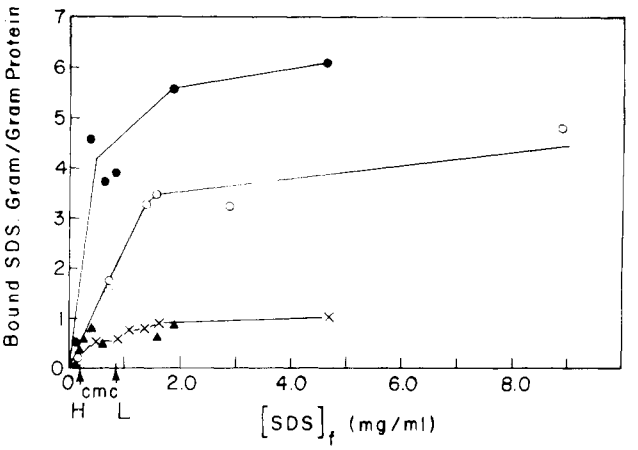

FIGURE 1: Binding of [^{35}S]$NaDodSO_4$ to the biochemically isolated sodium channel and bovine serum albumin. (●) Binding of $NaDodSO_4$ to sodium channel glycopeptide at high ionic strength; (○) sodium channel in low ionic strength; (▲) binding of detergent to BSA at high ionic strength; (×) BSA at low ionic strength. "H" and "L" show the critical micelle concentrations ("CMC") for $NaDodSO_4$ at the respective ionic strengths.

The eel sodium channel has been shown to have a 4-fold greater electrophoretic free mobility compared to standard proteins (Miller et al., 1983), thus preventing the use of $NaDodSO_4$–PAGE as a means to determine the molecular size of the channel peptide. This unusual behavior has been inferred to be due to the anomalously high binding of $NaDodSO_4$ to hydrophobic regions of the channel molecule, an inference well supported by the extensive literature on $NaDodSO_4$–protein interactions and their affect on $NaDodSO_4$–PAGE behavior [Pitt-Rivers & Impiombato, 1968; Reynolds & Tanford, 1970a,b; Grefrath & Reynolds, 1974; for further discussion, see Miller et al. (1983)].

$NaDodSO_4$ Binding by the Sodium Channel Glycopeptide. We have directly measured the binding of [^{35}S]$NaDodSO_4$ to the sodium channel at both high and low ionic strengths (Figure 1). Under both conditions, the channel polypeptide bound at least 4-fold more $NaDodSO_4$ than bovine serum albumin (BSA), a well-characterized protein which others have found to bind 1.4 and 0.4 g of $NaDodSO_4$/g of protein at low and high ionic strength, respectively (Reynolds & Tanford, 1970a). As explained above, the enhanced detergent binding shown in Figure 1 must be reflected in a commensurate increase in the electrophoretic driving force on $NaDodSO_4$–PAGE and would certainly account for the similarly elevated free mobility seen in Ferguson plots of the channel polypeptide. Thus, as for other proteins, it appears that the electrophoretic free mobility of the sodium channel peptide is a good index of its $NaDodSO_4$ binding capacity.

In addition, most of the elevated binding of $NaDodSO_4$ occurred well above the critical micelle concentration (CMC) for the detergent. This is also in contrast to standard proteins, such as BSA, which mainly interact with detergent monomers and bind saturating amounts of $NaDodSO_4$ just below the CMC (Reynolds & Tanford, 1970a). This suggests that the increased binding of $NaDodSO_4$ to the channel polypeptide

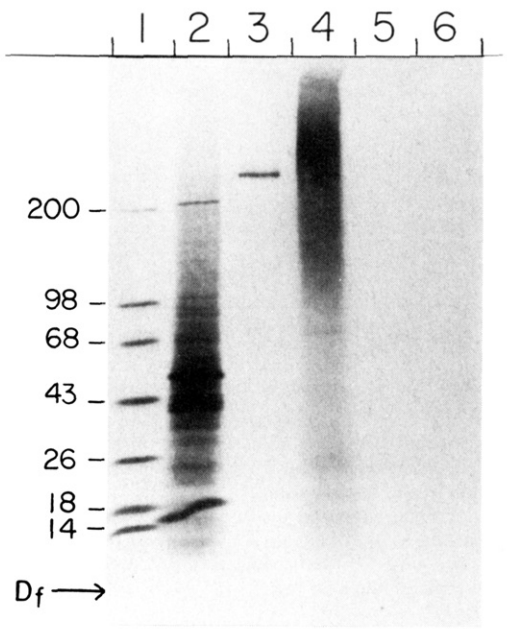

FIGURE 2: Autoradiograph of the [^{35}S]methionine-labeled proteins synthesized as a result of the addition of electroplax RNA to an in vitro translation system. The total proteins synthesized are shown in lane 2, while the polypeptide precipitated by anti-eel sodium channel antibody is shown in lane 3. Controls: lane 6, antibody preblocked with biochemically purified unlabeled sodium channel; lane 5, proteins synthesized by the reticulocyte lysate system in the absence of eel RNA. Lane 1, ^{14}C-labeled protein standards; lane 4, the ^{125}I-labeled native sodium channel (note the characteristic spreading of the band, accentuated here by overexposure). Samples were run on a 5–30% gradient NaDodSO$_4$–polyacrylamide gel with low cross-linking.

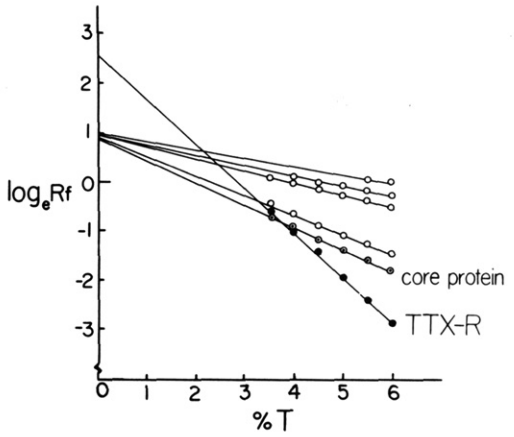

FIGURE 3: Ferguson analysis of the electrophoretic behavior of the core polypeptide synthesized in vitro. This is a plot of the natural logarithm of the relative electrophoretic mobility (R_f) vs. the acrylamide concentration used to cast the separating NaDodSO$_4$ gel (%T). ^{14}C-Labeled protein standards (○) from top to bottom were ovalbumin, bovine serum albumin, phosphorylase *b*, and myosin. The migration of the ^{35}S-labeled core polypeptide synthesized in vitro is shown by (⊙). The closed circles (●) show the migration of the peak (middle) of the ^{125}I-labeled native sodium channel band.

is through the ordering or binding of detergent micelles around very hydrophobic regions on the molecule [see Grefrath and Reynolds (1974)]. The extensive binding of micelles to the channel molecule is also supported by our observations that at high ionic strengths the amount of NaDodSO$_4$ bound to the protein is greater than at low ionic strengths (Figure 1). For standard proteins, such as BSA, the amount of detergent bound actually *decreases* with increasing ionic strength. This is because BSA, like the majority of other proteins, binds only detergent monomers, whose limiting free concentration decreases with the lowered CMCs at higher ionic strength (Reynolds & Tanford, 1970a). The most likely explanation for the behavior of the sodium channel protein is that increased detergent binding at high ionic strength is a result of increased size of the NaDodSO$_4$ *micelles* which are bound or organized by the polypeptide in high salt (Tanford, 1980).

Posttranslational Acquisition of Anomalous Electrophoretic Free Mobility. Although the amino acid composition of the sodium channel is not unusually hydrophobic (Miller et al., 1983), extensive sequences of hydrophobic residues are present on the molecule (Noda et al., 1984) and may be responsible for the ordering of micellar NaDodSO$_4$. Alternatively, the protein could have posttranslational modifications, for example, covalently bound fatty acids or phospholipids (Magee & Schlesinger, 1983; Schmidt, 1983), that are responsible for the increased binding of NaDodSO$_4$. To distinguish between these two possibilities, we have used a cell-free system to synthesize the sodium channel core polypeptide and test whether this molecule displays unusual hydrophobic characteristics in the absence of posttranslational processing. Eel electroplax total RNA was used to direct protein synthesis in a rabbit reticulocyte lysate system. The [^{35}S]methionine-labeled proteins that were synthesized ranged in apparent molecular mass from 12 to 200 kilodaltons when analyzed by NaDodSO$_4$–PAGE (Figure 2, lane 2). The eel sodium channel antisera specifically precipitated a polypeptide of M_r 230K from the translation mixture (Figure 2, lane 3). This peptide accounted for approximately 0.1% of the eel proteins that were synthesized, as estimated by comparing the radioactivity precipitated with channel antisera with the total radioactivity precipitated by trichloroacetic acid from the addition of electroplax RNA. In addition, the core polypeptide has a sharp banding pattern on NaDodSO$_4$–PAGE, which is in contrast to the diffuse banding pattern that is typical of the native sodium channel (compare lanes 3 and 4, Figure 2). The core polypeptide then was subjected to Ferguson analysis to determine whether it exhibited the unusually high electrophoretic free mobility characteristic of the mature channel molecule. As is clearly seen in Figure 3, the core polypeptide actually had a slightly *lower* free solution mobility than the standard proteins. Thus, a posttranslational modification of the core polypeptide was required before the mature channel polypeptide exhibited its high electrophoretic free mobility.

Taking the slightly lower free solution mobility (0.87 that of standards) into account allows us to estimate the molecular mass of the M_r 230K core protein as approximately 200 kilodaltons. This is in close agreement with the value obtained from both electron microscopy (Ellisman et al., 1982) and primary amino acid sequence data derived from cDNA clones of the channel gene (Noda et al., 1984).

Biosynthesis of Sodium Channel Peptides by Electrocytes. The large oocytes from *Xenopus laevis* have been used as a model system to investigate the translation of exogenously injected mRNAs and any subsequent processing of the newly synthesized polypeptides (Colman, 1984). In addition, Miledi and collaborators have injected mRNA from electrically excitable tissue into oocytes and have detected the presence of a number of voltage-sensitive ion channels via the two-electrode voltage clamp (Miledi et al., 1982). More recently, the frog oocyte expression system has been used by Numa and collaborators to investigate the electrophysiological expression of in vitro transcribed mRNAs from cDNA sequences to channel genes (Mishina et al., 1984; Noda et al., 1986b). Since the functional expression of eel sodium channels in the frog oocyte (as measured electrophysiologically) has met with apparent difficulty (Agnew, 1986), we have investigated the

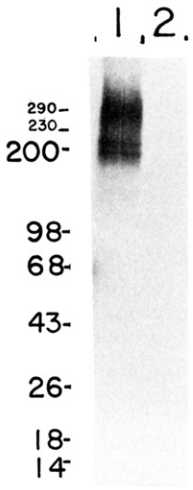

FIGURE 4: Autoradiograph of [^{35}S]methionine-labeled sodium channels synthesized in the eel electrocyte. Electrocytes were incubated in [^{35}S]methionine for 15 h, and the labeled channels were precipitated with specific antisera, as shown in lane 1. In lane 2, the antisera had been completely preblocked with the biochemically purified sodium channel.

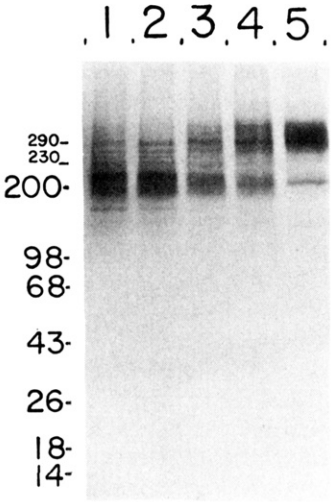

FIGURE 5: Autoradiograph of the [^{35}S]methionine-labeled sodium channels in the electrocyte that were chased with unlabeled methionine. Electrocytes were incubated in [^{35}S]methionine for 45 min (lane 1) and then chased with unlabeled methionine for 1, 3, 8, and 24 h, as shown in lanes 2, 3, 4, and 5, respectively. The labeled molecules were precipitated with specific antisera.

biochemical expression and posttranslational processing events that occurred as the eel sodium channel matured in both the eel electrocyte and the frog oocyte.

First, we have used the eel electrocyte to investigate the biosynthetic pathway the sodium channel takes in its parent tissue so we could compare it to the frog oocyte system. We incubated isolated electrocytes in [^{35}S]methionine for different periods of time and immunoprecipitated the radiolabeled channels with specific antisera. Incubation of electrocytes in [^{35}S]methionine for 15 h resulted in the appearance of diffusely banding polypeptides at M_r 200K, 210K, and 290K; these bands were not immunoprecipitated when the antisera were preblocked with biochemically isolated sodium channel (Figure 4). Next, electrocytes were incubated for 45 min in [^{35}S]-methionine and chased with unlabeled methionine, and a number of both tightly and diffusely banding polypeptides that ranged in molecular weight from 180K to 355K were immunoprecipitated (Figure 5). At the start of the chase, most of the label is associated with the diffuse bands at M_r 200K and 210K. After 24 h, most of the label chased to produce the

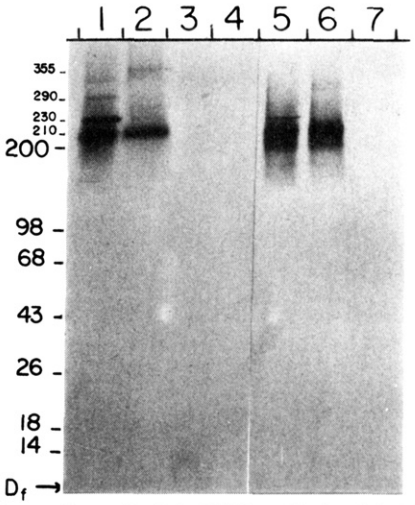

FIGURE 6: Autoradiograph of the [^{35}S]methionine-labeled eel sodium channel polypeptides synthesized in the frog oocyte in response to the injection of eel RNA. The polypeptides precipitated with antisera to the eel sodium channel after a 10- or 20-h incubation are shown in lanes 1 and 5, respectively. The glycosylated sodium channel polypeptides that were precipitated with WGA beads after a 10- or 20-h incubation are shown in lanes 2 and 6, respectively. Controls: lane 3, antisera preblocked with biochemically purified unlabeled eel sodium channel; lanes 4 and 7, sodium channel binding to WGA beads in the presence of 100 mM *N*-acetylglucosamine. The positions and molecular weights of the standard proteins, as well as the molecular weights of the sodium channel polypeptide bands, also are shown. The native eel sodium channel peak band would be at approximately M_r 290K on this gradient gel, which was as in Figure 2.

diffuse band at 290K characteristic of the mature native channel. The absence of a heavy band at M_r 230K, which would have corresponded to the core polypeptide seen in the cell-free system, suggested that the core polypeptide was rapidly posttranslationally processed in the electrocyte. The bands of greater and lesser molecular weight than the core polypeptide seen on $NaDodSO_4$–PAGE suggested that these were processed channel polypeptides.

Biosynthesis of Electroplax Sodium Channels by Xenopus Oocytes. We next investigated whether the frog oocyte could posttranslationally process eel sodium channels similarly to the electrocyte. Oocytes were injected with eel electroplax mRNA, or total RNA, and incubated in a medium containing [^{35}S]methionine for 10 or 20 h. As in the electrocyte system, specific antisera were used to precipitate the channel molecules from whole oocyte homogenates. After a 10- or 20-h incubation in [^{35}S]methionine, a number of radiolabeled polypeptides were specifically precipitated with sodium channel antisera (Figure 6, lanes 1 and 5). First, there was a prominent peptide of M_r 230K that was indistinguishable in molecular weight from the core polypeptide synthesized in the cell-free system. In addition, polypeptides of greater *and* lesser molecular weight than the core polypeptide were present, as was found in the electrocyte system. Most prominent among these were species of M_r 200K, 210K, 290K, and 355K.

The diffuseness of the banding patterns above and below the core polypeptide, as was found in the electrocyte, were suggestive of differential glycosylation or partial proteolysis. To assist in ascertaining which of the polypeptides were glycosylated, wheat germ agglutinin beads, were used to precipitate any channel glycopeptides containing the appropriate *N*-acetylglucosamine residues. The sharply banded, M_r 230K species was not precipitated by this lectin procedure, supporting its assignment as the unprocessed core protein seen in in vitro translation mixtures. The diffuse bands at M_r 210K and 200K and the bands at M_r 355K were specifically precipitated by

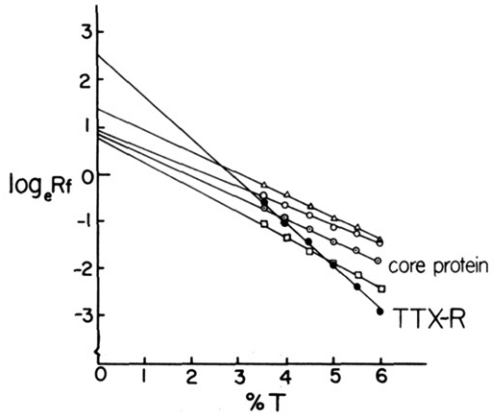

FIGURE 7: Ferguson analysis of the electrophoretic behavior of eel sodium channel polypeptides synthesized in the frog oocyte. The open circles (○) represent ^{14}C-labeled myosin. The triangles (△) represent the band at M_r 210K on the gradient gel, while the core polypeptide is represented by the (⊙) symbols. The major band above the core polypeptide at M_r 290K is represented by the (□) symbols. The closed circles (●) show the migration of the ^{125}I-labeled native sodium channel band.

WGA-beads (Figure 6, lanes 2 and 6).

After a 10-h incubation, there was a prominent band above the core polypeptide at M_r 290K that was not precipitated by WGA beads (Figure 6, lanes 1 and 2). Apparently, after a 20-h incubation, the band at M_r 290K was present in only trace amounts, while a new diffuse band appeared at M_r 200K that was only a small component at 10 h (Figure 6, lane 5). In addition, the core polypeptide comprised a greater proportion of the total radioactivity at 10 vs. 20 h. These data would suggest that the [^{35}S]methionine had been depleted from the amino acid pool, and thus, these results probably reflect a chase.

Identification of Biosynthetic Intermediates. What might be the relationship between the core polypeptide and the glycosylated bands with smaller molecular weight seen in both the electrocyte and the frog oocyte? This question is of interest since glycosylation greatly tends to *increase* the frictional coefficient of a peptide on NaDodSO$_4$–PAGE, thus *increasing* its molecular weight (Pitt-Rivers & Impiombato, 1968; Leach et al., 1980). It is possible that these species were smaller because they were proteolytic products of some glycosylated channel peptides; alternatively, these species may have *appeared* smaller on NaDodSO$_4$–PAGE due to the acquisition of the high electrophoretic free mobility characteristic of the processed native molecule. The latter possibility was assessed by using Ferguson analysis.

The diffuse band at M_r 210K exhibited a high electrophoretic free mobility, though not as great as the native channel isolated from electroplax membranes (Figure 7). The anomalous mobility of this component could also be seen relative to the myosin standard, since it ran slower than myosin (M_r 200K) on the gradient gel (Figure 6) but faster on the homogeneous gels used for Ferguson analysis (Figure 7). It was not possible to estimate accurately the position of the diffuse band at M_r 200K on the Ferguson gels due to extreme spreading of the band. Also shown in Figure 7 is the electrophoretic free mobility of the major 10-h band at M_r 290K as well as the core polypeptide. Both of these sharp bands had a slightly lower electrophoretic mobility than the standard proteins. There were insufficient amounts of the weak band at M_r 355K on the more porous (lower %T) Ferguson gels to accurately determine its R_f values and hence its exact electrophoretic mobility. However, this band did pass the band at M_r 290K on the gel as the acrylamide concentration was

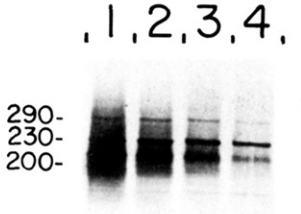

FIGURE 8: Autoradiograph of [^{35}S]methionine-labeled eel sodium channels in the frog oocyte that were chased with unlabeled methionine. Oocytes were incubated in [^{35}S]methionine for 16 h (lane 1) and then chased with unlabeled methionine for 20, 50, and 75 h, as shown in lanes 2, 3, and 4, respectively. The labeled molecules were precipitated with specific antisera.

lowered from 6% to 5%, indicating that it had a higher electrophoretic mobility than this band.

Will the major bands at M_r 200K and 210K be processed to the mature form of the sodium channel with longer incubation times? Oocytes were incubated in [^{35}S]methionine for 16 h and then chased with unlabeled methionine for up to 75 h (Figure 8). Apparently, the majority of the labeled peptides were not processed to the mature form of the sodium channel; instead, the bands weakened with time. However, there were some diffusely banding polypeptides at the approximate molecular weight of the native channel, and these may have been completely processed channels. We also performed shorter pulse/chase experiments, injected lesser and greater amounts of eel mRNA or total RNA, and recorded the same basic result (data not shown). In our hands, all of the oocyte batches that were injected produced the core polypeptide. However, different batches of oocytes varied in their ability to process this core molecule to the bands at M_r 200K and 210K. In a few cases, only the core polypeptide and bands with higher molecular weight were found.

Discussion

We have described the in vitro biosynthesis of the sodium channel core polypeptide from eel electroplax tissue as well as its biochemical expression in eel electrocytes and frog oocytes. Our data demonstrate that the unusual molecular properties exhibited by the native sodium channel on NaDodSO$_4$–PAGE are due to posttranslational modifications. While the diffuse banding pattern of the sodium channel on NaDodSO$_4$ gels has been inferred to be due to varying degrees of glycosylation of the core polypeptide (Miller et al., 1983; Agnew et al., 1983), we have presented evidence in this report suggesting that the high electrophoretic free mobility of the sodium channel on polyacrylamide gels is due to binding of large amounts of NaDodSO$_4$ to posttranslationally modified regions of the core polypeptide.

It was not possible to measure directly the binding of [^{35}S]NaDodSO$_4$ to the channel core polypeptide synthesized in the cell-free system, since only femtomole quantities were produced. However, as discussed under Results, the rather normal electrophoretic free mobility exhibited by the core polypeptide must be a consequence of its normal binding of NaDodSO$_4$. If, for example, the core polypeptide bound as much NaDodSO$_4$ as the mature molecule, one would expect it to have an electrophoretic free mobility *even greater* than that of the mature channel peptide. This is because the core polypeptide lacks the numerous carbohydrate residues on the mature molecule (Miller et al., 1983) that contribute significantly to the frictional properties of the polypeptide on PAGE but do not themselves bind NaDodSO$_4$ (Leach et al., 1980).

It is perhaps possible that the hydrophobic regions on the mature sodium channel are due to an unknown processing step

which causes the association of various hydrophobic amino acid sequences through unusual interactions such as non-sulfhydryl linkages. However, the most likely posttranslational modification that would be consistent with the binding of micellar $NaDodSO_4$ to the channel is the covalent attachment of fatty acids or phospholipids. In recent years, a number of membrane proteins have been shown to contain covalently attached fatty acids (Magee & Schlesinger, 1983; Schmidt, 1983). For the sodium channel, preliminary data indicate that the mature channel peptide is closely associated with about 8% fatty acyl moieties by weight after $NaDodSO_4$ gel filtration followed by extensive dialysis, while about 40% of this fatty acyl material remains associated with the protein even after further extraction with chloroform/methanol (Levinson et al., 1986a,b). These data support the hypothesis that the posttranslationally acquired hydrophobic domains on the mature channel are probably covalently attached lipids.

The electrocyte pulse/chase experiments demonstrate that the eel sodium channel has a very complex biosynthetic history. Care must be taken in assigning molecular weights to the intermediately processed channels on the basis of their apparent molecular weight on $NaDodSO_4$–PAGE, since the different glycopeptides will have different electrophoretic free mobilities. Early biosynthetic intermediates indentified from the electrocyte pulse/chase experiments had a lower molecular weight than the core polypeptide on $NaDodSO_4$ gels. After 45 min in [^{35}S]methionine, most of the radiolabeled channel peptides were the bands at M_r 200K and 210K. Additional processing steps apparently occurred relatively slowly, since it took an additional 24 h for all the label to be chased to the mature diffuse band at M_r 290K.

The *Xenopus* oocytes processed the majority of eel sodium channels to apparent intermediate forms identical in molecular weight with those identified in the electrocyte. As judged by lectin bead precipitation and Ferguson analysis, these peptides were partially glycosylated, with the main products migrating faster on gradient $NaDodSO_4$–PAGE (Figure 6) and displaying a higher electrophoretic free mobility (Figure 7) than the core polypeptide. These intermediates were capable of binding to WGA lectin, indicating that they had been partially processed in the medial Golgi apparatus (Dunphy et al., 1985). The further extensive addition of carbohydrate and hydrophobic domains to the channel, which are required before the channel acquires its mature physiochemical properties, presumably takes place in the medial or trans Golgi. In addition, the band at molecular weight 290K exhibited near-normal electrophoretic free mobility, indicating that it lacked hydrophobic domains. Only a small trace of this band was precipitated by WGA beads, suggesting either the absence of the appropriate *N*-acetylglucosamine residues on the molecule or their inaccessibility for lectin binding. This also may indicate that the molecule is a pre-Golgi, high-mannose-type glycoprotein, since it has been proposed that WGA binding competency is obtained only after processing by mannosidase and *N*-acetylglucosamine transferase in the medial Golgi (Dunphy et al., 1985).

As judged by $NaDodSO_4$–PAGE, the majority of eel sodium channels were not completely processed to the mature form in the frog oocyte. Furthermore, the disappearance of the major bands at M_r 200K and 210K during the pulse/chase experiments (Figure 8) suggested that these components were degraded before being posttranslationally processed further. We also recorded the same basic result when shorter pulses of labeled methionine were used or lesser amounts of either mRNA or total RNA were injected (data not shown). There are a number of examples in which the frog oocyte has failed to posttranslationally process an exogenous protein properly (Colman, 1984).

The length of the incubations required in this study also suggested that the channel polypeptide was processed relatively slowly in the frog oocyte. Recent electrophysiological investigations have suggested that the injection of eel electroplax mRNA into oocytes resulted in the appearance either of sodium channels on the cell surface after a 4-day incubation (Hirono et al., 1985) or of no channels at all (Agnew, 1986), in contrast to the readily observed expression of rat brain sodium channels (Gundersen et al., 1983; Noda et al., 1986b). Thus, our data suggest that slow and incomplete processing and degradation of the majority of eel sodium channels could well contribute to the low number or lack of channels on the oocyte surface. Depending on the degree of cell surface invaginations, one can estimate that about 10000 functional sodium channels would be required on the plasma membrane of an oocyte to give a measurable sodium current of about 10 nA. This is roughly 5 orders of magnitude *less* than the 10 fmol of all channel-related peptides we estimate that an oocyte was capable of producing in as little as 5 h. Thus, one can reasonably conclude that at best only a minute fraction of the synthesized eel sodium channels are being transported to the cell surface in a functional state.

A detailed study of the biosynthesis of rat brain sodium channels has recently appeared (Schmidt & Catterall, 1986). In the presence of tunicamycin, a molecule of M_r 203K was found, suggesting that this was the size of the core polypeptide of the brain α subunit that is homologous to the electrocyte peptide. This value is somewhat less than the molecular weight (230K) of the electrocyte core peptide, while the molecular weight of the brain α peptide deduced from cDNA sequence analysis is much larger than the electrocyte protein (228K vs. 208K; Noda et al., 1986a). In contrast to the electrocyte pulse/chase results, early biosynthetic intermediates from brain had a higher molecular weight than the core polypeptide. Thus, the earliest biosynthetic intermediate to the α subunit of the rat brain sodium channel was a glycosylated molecule of M_r 224K. This glycopeptide gradually increased in apparent size to the mature form at M_r 260K. These differences in the biosynthetic processing of the electroplax vs. the rat brain sodium channel may be due either to tissue or to species specificity. In view of the apparent differential expression of rat vs. eel sodium channels in frog oocytes, it would be of interest to determine how much of the varying synthetic history of different sodium channels is due to differences in amino acid sequence vs. differences in the biosynthetic apparatus of the parent tissue.

The voltage-regulated sodium channel appears to be an especially heavily posttranslationally modified protein. The role these modifications may play in channel distribution and function in electrically excitable tissue remains to be elucidated. The possible role played by the negatively charged carbohydrates in channel conductance and gating has been discussed previously (Miller et al., 1983). The data presented in this report support the hypothesis that the channel acquires extensive hydrophobic domains during its synthesis, very possibly covalently attached lipids. The role played by the covalent attachment of hydrocarbon chains to membrane proteins is still speculative. Possible functions include the anchoring of the protein in the membrane or as a signal for protein sorting within membranes (Schlesinger & Malfer, 1982). It also has been suggested that this posttranslational modification may play a role in subunit assembly of some receptors in the Golgi

apparatus and their cell surface expression (Olson et al., 1984). For the sodium channel, the voltage sensors that control channel gating are thought to lie in a hydrophobic lipid domain surrounding the molecule (Hille, 1984). Thus, it will be of interest to determine if the extensive hydrophobic domains demonstrated in the present study might play a role in the molecular mechanism of the sodium channel itself.

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