

RAPID COMMUNICATIONS

NEUROFILAMENT PROTEIN IS PHOSPHORYLATED IN THE SQUID GIANT AXON

HARISH C. PANT, GORDON SHECKET, HAROLD GAINER, and RAYMOND J. LASEK. From the Laboratory of Neurobiology, National Institute of Mental Health, Bethesda, Maryland 20014, the Laboratory of Developmental Neurobiology, National Institute of Child Health and Human Development, Bethesda, Maryland 20014, the Neurobiology Center and Department of Anatomy, School of Medicine, Case Western Reserve University, Cleveland, Ohio 44106, and the Marine Biological Laboratory, Woods Hole, Massachusetts 02543

ABSTRACT

We have observed the phosphorylation of neurofilament protein from squid axoplasm. Phosphorylation is demonstrated by ^{32}P labeling of protein during incubation of axoplasm with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. When the labeled proteins are separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), two bands, at 2.0×10^5 daltons and $>4 \times 10^5$ daltons, contain the bulk of the ^{32}P . The 2.0×10^5 -dalton phosphorylated polypeptide comigrates on SDS-PAGE with one of the subunits of squid neurofilament protein. Both major phosphorylated polypeptides co-fractionate with neurofilaments in discontinuous sucrose gradient centrifugation and on gel filtration chromatography on Sepharose 4B. The protein-phosphate bond behaves like a phospho-ester, and labeled phospho-serine is identified in an acid hydrolysate of the protein. The generality of this phenomenon in various species and its possible physiological significance are discussed.

KEY WORDS neurofilaments · 10-nm filaments · neurofilament protein · phosphoproteins · axoplasm

Recently, Pant and Yoshioka described the phosphorylation of proteins in axoplasm of the squid giant axon (9). Phosphorylation was demonstrated either by intracellular injection of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ into giant axons, or by addition of $[\text{}^{32}\text{P}]\text{ATP}$ to axoplasm which was isolated from the axons by extrusion. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of the phosphorylated proteins demonstrated that the pattern of labeling was simple and that two polypeptide bands contained most of the incorporated ^{32}P . One of the polypeptides has a mol wt 2.0×10^5 daltons, and the

other has a mol wt $>4 \times 10^5$ daltons, barely entering a 7.5% gel.¹

Lasek and Hoffman (7) and Gilbert (1) have shown that the two major subunits of squid neurofilament protein (NFP) have molecular weights by SDS-PAGE of 2.0×10^5 and 6.0×10^4 daltons. The subunits of NFP were identified by purifying the intact neurofilaments away from the other major constituents of extruded axoplasm. Neurofilaments were identified by their characteristic fine structure as seen with the electron microscope. The standard methods developed to purify

¹ Pant, H. C., T. Yoshioka, I. Tasaki, and H. Gainer. 1978. Divalent cation dependent phosphorylation of proteins in the squid giant axon. Submitted to *Brain Res.*

neurofilaments from axoplasm (7) involved: homogenization of axoplasm in buffer containing 0.1 M salt, reducing agent, and a Ca^{++} chelator; differential centrifugation; sedimentation through a discontinuous sucrose gradient; and gel filtration on Sepharose 4B in a 0.1 M KCl buffer. The 2.0×10^5 and 6.0×10^4 -dalton polypeptides were present in all fractions that contained neurofilaments and were the major constituents of highly purified neurofilaments. Using a gradient gel SDS-PAGE procedure capable of resolving very high molecular weight polypeptides, we have recently observed that a polypeptide of $>4 \times 10^5$ daltons is also consistently present in purified neurofilament preparations in substantial amounts. We suspect that this polypeptide is also a neurofilament subunit, but further study is needed to make this identification. (This polypeptide is referred to as "band 1" in Fig. 2).

The correspondence in electrophoretic mobilities of the 2.0×10^5 -dalton subunit of squid NFP and one of the two principal phosphorylated proteins in squid axoplasm suggested that this phosphorylated component is the same protein as the 2×10^5 -dalton subunit of NFP. We have tested this hypothesis by determining whether the ^{32}P -labeled protein co-fractionates with NFP, using the procedure established for purifying NFP, and have confirmed it. We have also characterized the properties of the bond between ^{32}P and the protein.

MATERIALS AND METHODS

Live squid were obtained from the Supply Department (Marine Biological Laboratory, Woods Hole, Mass.). They were kept in a seawater tank, and used within 48 h of capture. Dissection of giant axons and extrusion of axoplasm were performed as described previously by Lasek (6).

The phosphorylation reaction was begun by mixing 100 μl of freshly dissected axoplasm with 50 μCi of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (100 Ci/mmol in 50 μl of H_2O , obtained from New England Nuclear (Boston, Mass.)). The mixture was incubated for 40 min at 20°C , and the reaction was stopped either by cooling to 0°C or by adding an equal volume of 20% trichloroacetic acid (TCA).

Samples used for SDS-PAGE and for phospho-serine and phospho-threonine analysis were precipitated with an equal vol of 20% TCA. The precipitates were sedimented at 5,000 g for 10 min, washed five times with 1 ml of 10% TCA, and finally washed with 1 ml of ethanol.

Neurofilament-enriched fractions were prepared as follows. The axoplasm- ^{32}P ATP reaction mixture was homogenized in 0.3 ml of 0.15 M KCl, 0.5 mM dithio-

threitol (DTT), 0.5 mM ethylene glycol-bis(β -aminoethyl ether) N,N,N',N' -tetraacetate (EGTA), 30 mM imidazole pH 7.0 (buffer A), then centrifuged at $10^4 g$ for 20 min. The pellet was re-homogenized in 0.2 ml of buffer A and again centrifuged at $10^4 g$ for 20 min. The supernates were combined, and layered onto a step sucrose gradient (50 μl of 2.5 M, 150 μl of 0.5 M sucrose in buffer A) in a 0.65-ml tube, and centrifuged at $10^5 g$ for 2 h. The material collecting at the 0.5 M/2.5 M interface is enriched in neurofilaments and is designated "light neurofilaments" (LNF). The $10^4 g$ pellet was rehomogenized and layered onto a (50 μl of 2.5 M, 200 μl of 1.0 M, 200 μl of 0.5 M sucrose in buffer A) gradient, and centrifuged at $10^5 g$ for 2 h. The 1.0 M/2.5 M interface is also enriched in neurofilaments and is designated "heavy neurofilaments" (HNF). The $10^5 g$ supernates were combined.

TCA-precipitated samples for SDS-PAGE were redissolved in 50 μl of 6 M urea, 0.1% SDS, 5% mercaptoethanol, then heated to 100°C for 2 min. The procedure of Neville (8) was used for the 5% gel in Fig. 1. The procedure of Laemmli (5) was used for the 5-17.5% gradient gel in Fig. 2. Approx. 50 μg of protein were loaded onto each gel. The molecular weight standards were: rabbit myosin (2.0×10^5 daltons), bovine phosphorylase A (9.0×10^4 daltons), bovine serum albumin (6.8×10^4 daltons), rabbit actin (4.6×10^4 daltons),

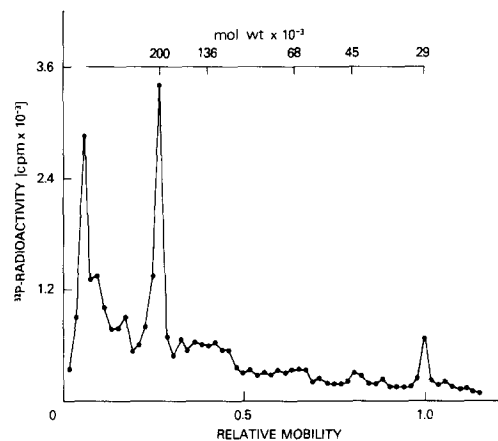


FIGURE 1 Molecular weight distribution of ^{32}P -labeled proteins from extruded squid axoplasm after electrophoresis on a 5% acrylamide SDS gel. Whole axoplasm extruded in a solution containing 150 mM NaCl, 1 mM MgCl_2 , and ^{32}P ATP was incubated for 30 min at 20°C . The reaction was stopped by the addition of TCA to a final concentration of 10%. The TCA pellet obtained by centrifugation was washed with ethanol-ether (1:1, vol/vol), and ether, and then solubilized in buffer containing SDS for electrophoresis. Note that the labeling pattern of whole axoplasm is dominated by two high molecular weight peaks which correspond to band 1 and NF200 (see Fig. 2 and text).

and squid tubulin prepared according to Shelanski et al. (11).

Gel filtration chromatography was carried out using a 0.9×25 cm column of Sepharose 4B, eluted with 0.6 M KI, 0.5 mM DTT, 0.5 mM EGTA, 30 mM imidazole pH 7.0. The void and inclusion volumes were determined with blue dextran and ATP, respectively.

RESULTS

The pattern of labeling of axoplasmic proteins by incubation of axoplasm with [32 P]ATP is shown quantitatively in Fig. 1. Labeled proteins from whole axoplasm were electrophoresed on a 5% acrylamide gel, which was sliced and counted for 32 P. Only two heavily labeled regions of the gel are seen. In Fig. 2, the labeled polypeptides in the neurofilament-enriched fraction LNF are shown by autoradiography after electrophoresis on slab gels. Although numerous stained bands are present, only two heavily labeled bands, one at $>4 \times 10^5$ and another at 2.0×10^5 daltons are seen. These correspond exactly with the stained "band 1" and "NF200." Since microtubule-associated proteins (MAPs) and tubulin are known to be phosphorylated (12), squid brain tubulin (containing MAPs) was included as a standard in the electrophoresis. Neither of the major labeled bands corresponds to MAPs. When the autoradiograph was exposed longer, a number of minor bands appeared, including the two tubulin subunits. The region of the gel containing MAPs was obscured by grains from band 1, so the possible presence of a small amount of label in this protein could not be observed. Even in the overexposed autoradiograph, there was no visible labeled band corresponding exactly with the 6.0×10^4 -dalton NFP subunit, NF60.

32 P-labeled axoplasmic protein was fractionated by the centrifugation scheme described above for purification of neurofilaments, in order to determine whether the major labeled proteins behave like NFP in this procedure. In this fractionation, a portion of the NFP sediments as highly polymerized neurofilaments, while the majority of the NFP remains in the high-speed supernate. The polymerized filaments are found in two morphologically distinct fractions, HNF which are aggregated into clumps, and LNF which are dispersed.² The sedimentation of the two major 32 P-labeled

² Shecket, G. and R. J. Lasek. 1978. A Mg^{++} or Ca^{++} activated ATPase from squid giant fiber axoplasm: possible association with neurofilaments. Submitted to *J. Neurochem.*

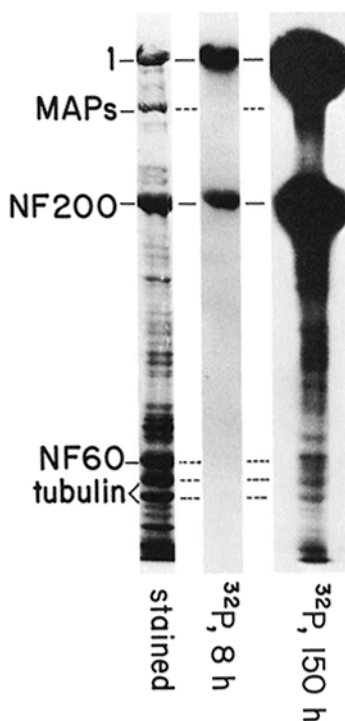


FIGURE 2 Demonstration of phosphorylated neurofilament proteins by SDS-polyacrylamide gel electrophoresis and autoradiography. Axoplasmic proteins were labeled with [32 P]ATP, then neurofilament-enriched fraction LNF was prepared, as described in Materials and Methods. A sample of LNF was run on a 5–17.5% acrylamide gradient slab gel. *Left:* The proteins are shown by staining with Coomassie Blue. *Middle:* After drying, the gel was applied to Kodak Royal X-omat film for 8 h; the film was developed in an X-omat processor, showing the pattern of 32 P-labeling. *Right:* The same gel was re-exposed for 150 h to show minor labeled bands. Polypeptides NF200 (2.0×10^5 daltons) and NF60 (6.0×10^4 daltons) have been previously identified as neurofilament subunits. Band 1 ($>4 \times 10^5$ daltons) is probably also a neurofilament subunit (see text). Note the absence of labeling of NF60. Microtubule protein prepared from squid brain by cyclic repolymerization of microtubules was run on similar slab gels along with LNF. The bands labeled "tubulin" and MAPs comigrate with the tubulin and MAPs of this standard.

axoplasmic proteins was entirely consistent with that of NFP; a portion of the labeled protein was found in each of the neurofilament-enriched fractions, while several-fold more labeled protein remained in the high-speed supernate.

An unusual property of the 2.0×10^5 -dalton NFP subunit and the $>4 \times 10^5$ -dalton polypeptide

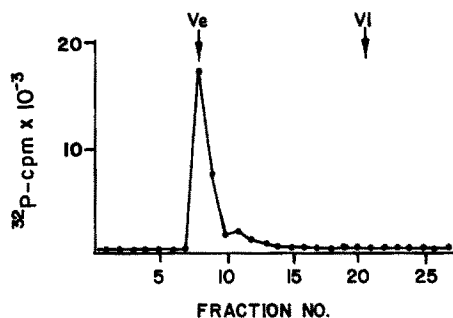


FIGURE 3 Elution profile of ^{32}P -labeled LNF on Sepharose 4B gel filtration chromatography. Axoplasmic proteins were labeled with [^{32}P]ATP, fraction LNF was prepared, and then a sample of labeled LNF was applied to a 0.9×25 cm column of Sepharose 4B, eluted with a buffer containing 0.6 M KI (see Materials and Methods). The profile of ^{32}P shown is representative of the TCA-precipitable ^{32}P in each column fraction. The exclusion volume V_e was determined with blue dextran, and the inclusion volume V_i was determined with ATP.

is that they remain in their high aggregate molecular weight form in the presence of 0.6 M KI, as demonstrated by their elution in the void volume on Sepharose 4B gel filtration chromatography with 0.6 M KI buffer.² When ^{32}P -labeled LNF was subjected to the same gel filtration separation, nearly all of the TCA-precipitable radioactivity appeared in the void volume, as shown in Fig. 3. Analysis of the voided material by SDS-PAGE showed that both the 2.0×10^5 - and the $>4 \times 10^5$ -dalton labeled species were present. The two ^{32}P -labeled polypeptides thus behaved exactly like the NF200 subunit of NFP and the band 1 polypeptide.

The nature of the phosphate-protein bond in the ^{32}P -labeled protein was studied by determining the fraction of ^{32}P which remained precipitable in cold 10% TCA after the following treatments: 10% TCA, 90°C, 20 min; 0.5 N NaOH, 90°C, 10 min; and 0.66 M hydroxylamine, 0.05 M acetate pH 5.4, 20°C, 20 min. 62% of the label was stable in hot TCA, 5% in hot alkali, and 89% in hydroxylamine. Thus, the bulk of the label behaves like phosphoester-bonded phosphate, which is stable in acid and hydroxylamine and labile in alkali, and unlike acyl phosphate, which is cleaved by hydroxylamine, or phosphoramidate phosphate, which is stable in alkali but labile in acid (13). Labeled protein was partially hydrolyzed (6 N HCl, 110°C, 2 h) and assayed for phospho-seryl and phospho-threonyl residues by high-voltage

paper electrophoresis, as described by Kirchner et al. (4). The partial hydrolysis conditions were chosen as a compromise between peptide hydrolysis and phosphoester hydrolysis. 41% of the ^{32}P migrated as orthophosphate ($R_f = 1.0$, i.e., positive electrode), 28% corresponded exactly with the phosphoserine standard ($R_f = 0.74$), 13% migrated at $R_f = 0.30$ as an identified peak, and 14% remained at the origin; no phospho-threonine peak was present. The majority of the label which was not hydrolyzed to orthophosphate was recovered as phospho-serine.

DISCUSSION

One of the two major phosphorylated polypeptides of squid axoplasm has been shown to behave exactly like the 2.0×10^5 -dalton subunit of squid neurofilament protein in three different separation procedures: SDS-PAGE, a centrifugation fractionation, and gel filtration on Sepharose 4B in 0.6 M KI. Thus, we conclude that the 2.0×10^5 -dalton phosphorylated protein of squid axoplasm is in fact the 2.0×10^5 -dalton NFP subunit NF200. The other major phosphorylated species, a $>4 \times 10^5$ -dalton polypeptide, also co-fractionates with NFP in the centrifugation and gel filtration separations, and corresponds on SDS-PAGE with a high molecular weight polypeptide, band 1, which is consistently present in squid neurofilament preparations. Band 1 may be a subunit polypeptide of neurofilament, but this remains to be proven.

Of the two polypeptides that have been clearly identified as subunits of squid neurofilaments, NF200 and NF60, only NF200 is phosphorylated. Labeled NF60 was not detectable even when the autoradiographs were grossly overexposed. This observation suggests that these two polypeptides are functionally different. The subunits also differ with regard to their behavior in gel-filtration chromatography on Sepharose 4B in the presence of 0.6 M KI. Under these conditions, the 2.0×10^5 -dalton subunit elutes in the void volume and is separated completely from the 6.0×10^4 -dalton subunit,² while both subunits elute in the void volume when a 0.1 M KCl buffer is used.³ The identification of such differential properties of neurofilament subunit proteins may assist in sort-

³ Lasek, R. J., N. Krishnan, and I. Kaiserman-Abrahamoff. 1978. Identification of the subunit proteins of 10 nm neurofilaments isolated from axoplasm of Squid and *Myxicola* giant axons. Submitted to *J. Cell Biol.*

ing out the multiple proteins found in preparations of neurofilaments from other species.

Phosphorylation appears to be a general property of neurofilaments. Experiments on the axoplasm of the giant axon of the marine polychaete *Myxicola* have shown that *Myxicola* NFP is phosphorylated. *Myxicola* NFP consists of two major polypeptides with mol wt 1.6×10^5 and 1.5×10^5 daltons (2, 7). Both proteins are phosphorylated, and they are the major phosphorylated components of the axoplasm, just as in the case of the squid (10). Mammalian neurofilaments apparently contain three major polypeptides with mol wts 2.0×10^5 , 1.45×10^5 , and 6.8×10^4 daltons. These polypeptides, first identified in axonal transport, have been called the neurofilament triplet (3). All three triplet polypeptides are phosphorylated in extracts of guinea pig nerve (10). Thus, we conclude that phosphorylation is a general physiological property of neurofilaments, having been found in *Mollusca*, *Annelida*, and *Vertebrata*. It will be of interest to determine whether 10-nm filaments from other cell types, such as fibroblasts, are phosphorylated.

The finding that the phosphate-protein bond in squid NFP behaves like a phosphoester bond and the demonstration of phospho-serine in the hydrolysate of NFP prove that the label is covalently bound to the protein. The phospho-serine localization of the protein-bound phosphate is typical of proteins that are acted upon by protein kinases in systems where phosphorylation is known to specifically modify enzymatic activities of the phosphorylated proteins. The functional role of neurofilaments in the axon is poorly understood at present. The observation that NFP is the principal phosphorylated protein in the axon suggests that NFP has some active interrelationships with other proteins of the axon, possibly under metabolic control by a protein kinase-protein phosphatase system. Further study of this phenomenon promises to offer clues to the functional role of neurofilaments in the physiology of the axon.

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