Clustering of Phosphorylated Amino Acid Residues in Neurofilament Proteins As Revealed by ^{31}P NMR†

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ABSTRACT: The state of phosphorylation in neurofilament (NF) proteins is studied by the ^{31}P NMR technique. The ^{31}P NMR spectrum of intact NF proteins at pH 7.0 is comprised of a major resonance at 4.18 ppm and a minor resonance at 3.53 ppm. The chemical shifts of the major and minor resonances are strongly dependent on pH and have pK_a values for phosphoserine of 5.85 and for phosphothreonine of 6.00, respectively. ^{31}P NMR spectra of isolated NF polypeptides show nonequivalent phosphoserine clusters in NF150 and in NF200. Their chemical shifts are very similar in both polypeptides, but the intensities of homologous resonances are different. NF68 has no detectable ^{31}P resonance signal. Phosphate-specific monoclonal antibodies to NF200 can distinguish phosphates of various clusters. Microtubule proteins can also produce specific alteration of the ^{31}P resonances of NF200. NF proteins digested by calcium-activated neutral protease (CANP) show relatively little change in ^{31}P resonances.

Neurofilaments (NFs) are a major structural component of the neuronal cytoskeleton and serve as an important determinant of neuronal size and shape. Mammalian NFs consist of three polypeptides with M_r of 200 000 (NF200), 150 000 (NF150), and 68 000 (NF68) when analyzed by sodium dodecyl sulfate (SDS) gel electrophoresis. All three polypeptides, like all other intermediate filament protein subunits, possess a central "rod" domain (M_r 40 000), which is rich in α-helical configuration and forms the backbone (or core) of the assembled filament. The adjoining COOH-terminal segments protrude from the filament core and are structurally unique among the three polypeptides, increasing in size from NF68 to NF200 (Geisler et al., 1983, 1985). The COOH-terminal segment of NF200 may form part of the cross-bridges between neighboring NFs and microtubules (Hirokawa et al., 1984).

Large amounts of phosphate are present in NF150 and NF200 (Jones & Williams, 1982), especially in their COOH-terminal segments (Julien & Mushynski, 1983). NF phosphate occurs mostly as phosphoserine, but smaller amounts of phosphothreonine have also been detected in NF150 (Julien & Mushynski, 1982). There is some evidence that the phosphorylated residues are present in a restricted area of both polypeptides (Julien & Mushynski, 1983), specifically in the inner region of the COOH-terminal segment adjacent to the central rod domain (see schematic diagram, Figure 1A). Phosphate groups of NFs can be readily removed by phosphatases (Julien & Mushynski, 1983), indicating their external localization within assembled NFs. Further, the phosphates are recognized as the important antigenic loci in NF proteins (Sternberger & Sternberger, 1983; Carden et al., 1985; Lee et al., 1986).

Currently, it is not known if NF phosphorylation is of functional importance. Increased amounts of phosphorylation occur on NFs as they are transported from the perikarya into axons (Bennett & DiLullo, 1985). In addition, differing states of phosphorylation occur among populations of neurons (Lee et al., 1986). Phosphorylation has also been implicated in stabilizing NF assembly (Wong et al., 1984).

This study reports, for the first time, the ^{31}P NMR spectra of intact NFs and of NF polypeptides that have been separated by SDS gel electrophoresis. The identities and states of the phosphorylated amino acids have been established from the chemical shifts of resonances and from pH titration behaviors. The ^{31}P NMR spectra reveals the presence of several environmentally nonequivalent phosphate groupings in close proximity in NF proteins. Perturbation of ^{31}P resonances by biological ligands, such as monoclonal antibodies, microtubule proteins, and calcium-activated protease, discriminated further the various phosphate clusters in regard to their function and probable location within the NF structure.

Materials and Methods

NF proteins were purified from bovine spinal cord by methods described previously (Zimmerman & Schlaepfer, 1982) and were stored at −20 °C either as intact NFs or after solubilization and centrifugation in 8 M urea. Individual NF proteins were obtained by preparative electrophoresis using 7.5% SDS–polyacrylamide (3 mm thick) gels. Bands corresponding to the three major NF polypeptides were excised and equilibrated in water for 24 h to elute each polypeptide from the gel matrix. Eluted protein solutions were lyophilized, solubilized in a small amount of water, dialyzed extensively against water, and either used immediately or lyophilized and stored at −20 °C.

Polyclonal and monoclonal antibodies to bovine NF proteins were from previous studies (Lee et al., 1982). Antibodies were pretested for their capability to distinguish native (phosphorylated) NF proteins and enzymatically dephosphorylated forms in vitro by immunoblot studies (Carden et al., 1985). Microtubules were prepared from bovine brain by two successive cycles of assembly and disassembly (Williams & Lee, 1982). Calcium-activated neutral protease (CANP) with a low affinity for calcium was prepared as described (Zimmerman & Schlaepfer, 1984). All of the above protein preparations were dialyzed against 50 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (Hepes) buffer

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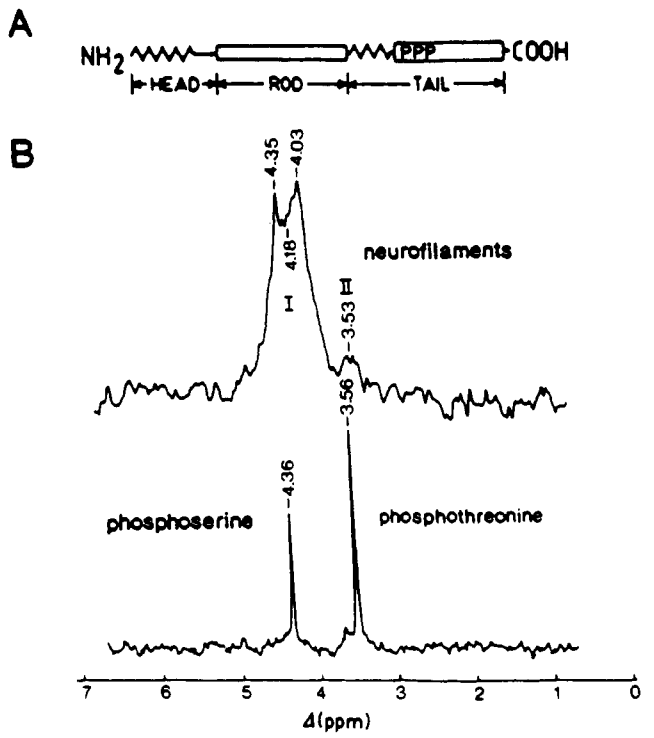

FIGURE 1: (A) A schematic diagram of NF polypeptide (Geisler et al., 1985). (B) (Top) ^{31}P NMR spectra of NF proteins, 2.8 mg/mL in 50 mM Hepes buffer containing 10 mM EDTA and 20% D_2O, pH 7.0, 35 644 scans. (Bottom) ^{31}P NMR spectra of phosphoserine (1 mM) and phosphothreonine (2 mM), added to above NF protein solution, 1500 scans.

containing 10 mM ethylenediaminetetraacetic acid (EDTA), pH 7.0, before use for a NMR experiment. The Hepes buffer and equivalent amounts of antibodies, microtubule proteins, and CANP were separately scanned for ^{31}P NMR signals prior to their additions to NF samples.

For experiments where pH was varied, NF proteins were dialyzed against Hepes buffer adjusted to various pHs. The pH of NF proteins was measured immediately before and after each spectrum was recorded.

^{31}P NMR measurements were made on neurofilament proteins in 1.5 mL of Hepes buffer, which were placed in 10-mm precision tubes (Wilmad) equipped with a Teflon vortex plug. The samples contained 20% D_2O as an internal field frequency lock and 10 mM EDTA to eliminate line broadening due to paramagnetic impurities. ^{31}P NMR spectra were measured at 22 °C with a Bruker WH-360 spectrometer at 145.732 MHz with quadrature phase detection. All spectra were recorded with broad-band proton decoupling. The typical conditions of the NMR experiment were 16K memory size, 2500-Hz sweep width, 35-μs pulse width, and 1.638-s acquisition time. Exponential multiplication of the free induction decay signal was applied to reduce noise with resulting line broadening of 10 Hz. Data represent, on the average, 35 000–50 000 accumulated scans. All chemical shifts were determined relative to an external standard of 85% H_3PO_4. Downfield shifts are given in a positive sign.

RESULTS

^{31}P NMR Spectrum of Intact NFs. Figure 1B (top trace) shows that the ^{31}P NMR spectrum of intact NFs in Hepes buffer, pH 7.0, is comprised of a major resonance (I) at 4:18 ppm and a minor resonance (II) at 3.53 ppm with 85% H_3PO_4 as a standard. The ^{31}P NMR spectra of phosphoserine and phosphothreonine derived under the same conditions are shown in the lower tracing. Dephosphorylation of NFs by incubation with *E. coli* alkaline phosphatase eliminated all of the ^{31}P NMR resonances.

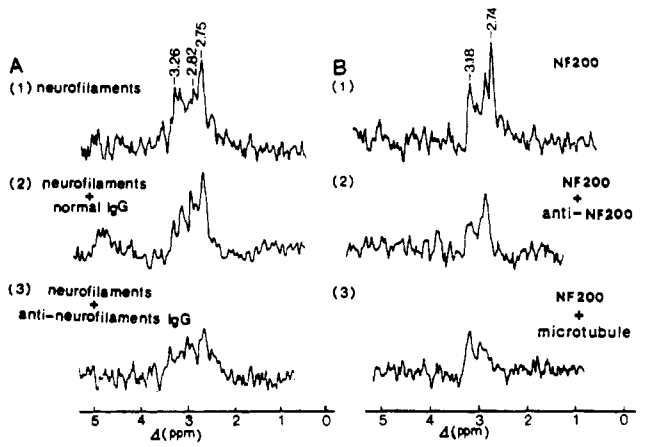

FIGURE 2: Perturbation of ^{31}P NMR spectra of NF proteins by various biological probes. (A) (1) NF proteins, 2.3 mg/mL, 35 500 scans; (2) NF proteins, 2.3 mg/mL, plus 10% (v/v) rat normal IgG, 34 645 scans; (3) NF proteins, 2.3 mg/mL, plus 10% (v/v) anti-NFs IgG, 43 502 scans. (B) (1) NF200, 2 mg/mL, 51 770 scans; (2) NF200, 2 mg/mL, plus monoclonal anti-NF200, 42 502 scans; (3) NF200, 2 mg/mL, plus microtubule proteins, 0.12 mg, 57 159 scans. All the samples were at pH 6.3–6.6.

^{31}P NMR of NF Proteins as a Function of pH. Maximum resolution of ^{31}P resonance was observed at pH 6.3–6.6 when the pH of NF protein solutions was varied from 4.0 to 9.0. The titration behavior of the individual components resolved at this pH could not be traced throughout the entire pH range. The major resonance shifted from 0.95 to 4.66 ppm while the minor resonance shifted from 0.62 to 4.19 ppm as the pH was increased from 4.0 to 9.0. The change in chemical shifts of approximately 4 ppm over the pH range 4.0–9.0 is almost identical with that shown by phosphomonoesters (Matheis & Whitaker, 1984). Furthermore, the major and minor resonances exhibited similar titration behaviors and pK_a values of 5.85 and 6.00, respectively, to those of free phosphoserine and phosphothreonine (Vogel & Bridger, 1983).

^{31}P NMR Spectra of Various NF Proteins. The ^{31}P resonances of urea-treated NF proteins show many more subpeaks than intact NF (data not shown). The greater complexity of the spectrum in urea indicates that some phosphomonoesters are shielded as a result of the polymerization of subunits under physiological conditions.

The ^{31}P resonance spectrum of NF150 contains a major resonance partially resolved at 3.21, 3.01, and 2.87 ppm and a minor resonance at 2.65 ppm. The ^{31}P resonance spectrum of NF200 also consisted of a major resonance comprised of partially resolved peaks at 3.18, 3.06, 2.88, and 2.74 ppm, and a minor resonance at 2.60 ppm. No ^{31}P resonance signal was observed with NF68 under similar conditions. The chemical shifts of the major and minor resonances of NF150 and those of NF200 were very similar while the intensities of the homologous resonance peaks were different. The 2.74 ppm resonance, which is most intense in NF200, is absent in NF150.

Perturbation of ^{31}P NMR Spectra of NF Proteins with Biological Probes. Addition of anti-NF antibodies (IgG) to preparations of intact NFs caused a marked alteration of the ^{31}P spectrum resulting in a greater complexity and substantial broadening of resonance signals (Figure 2A, part 3). In contrast, an equivalent addition of nonspecific IgG to NF proteins produced only a moderate broadening of the resonance signal with retention of the overall character of the ^{31}P NMR

spectrum (Figure 2A, parts 1 and 2). The specific antibody binding, therefore, appeared to be directed to the phosphorylated region of NF proteins.

A monoclonal antibody (4.3F9), known to recognize a phosphate site in the COOH-terminal region of NF200 (Carden et al., 1985), was used in order to identify a specific phosphate group or groups involved in the antibody binding. Addition of 4.3F9 antibody to NF200 caused coalescence of the pairs of downfield (3.18 and 3.06 ppm) and upfield (2.88 and 2.74 ppm) resonances (Figure 2B, part 2). A complementary antibody binding site for a continuous epitope, such as the phosphates of NF proteins, consists of five to eight amino acid residues (Wethoff et al., 1984). The data indicate that there are two pairs of phosphate clusters in NF200. A pair of phosphate cluster is set apart from the other by more than eight amino acids. On the other hand, phosphate clusters in a given pair (3.18 and 3.06 ppm or 2.88 and 2.74 ppm) are sufficiently close as to be within the antibody binding range.

Alterations in the ^{31}P resonance spectrum of NF200 was also brought about by the addition (1% w/w) of a microtubule preparation (containing MAPs proteins) from bovine brain. The upfield (2.88 and 2.74 ppm) resonances of NF200 were greatly affected by the addition of microtubule proteins (Figure 2B, part 3). A substantial reduction in the 2.74 ppm resonance peak height is particularly noteworthy, since this resonance was highly intense and was present only in NF200.

Proteolysis of NFs by calcium-activated neutral protease CANP caused extensive (>90%) degradation of NF proteins when examined by SDS–PAGE [see Schlaepfer et al. (1985)]. In contrast, the ^{31}P NMR spectrum of degraded proteins was not significantly different from that of NF proteins prior to proteolysis.

Discussion

This study has demonstrated that the ^{31}P NMR technique can be used to probe and characterize the nature of phosphate groups in isolated NFs. NF proteins contain 65–98 nmol of phosphate/mg of protein (Jones & Williams, 1982). The relatively high phosphorus contents of NF200 (1.5%) and NF150 (0.5%) are comparable to that of casein (0.2–1.3%) (Brauer & Sykes, 1984). Furthermore, NF polypeptides are highly stable and, hence, suited for the prolonged periods of measurements required to accumulate ^{31}P NMR signals.

The multiple components within the major resonance at pH 6.3–6.6 and the synchronous dependence of their chemical shifts with pH indicate that phosphate groups are in clusters within NF structure. Similar multicomponent ^{31}P NMR signals are observed in other phosphoproteins, e.g., casein, in which the phosphate groups are known to occur in clusters (Sleigh et al., 1983). Clustering of phosphoesters within the COOH-terminal regions of NF150 and NF200 has also been proposed on the basis of the chemical analyses of components from NF fragmentation (Julien & Mushynski, 1983; Geisler et al., 1985).

The sequencing of NF polypeptides has been partially completed (Geisler et al., 1985; Lewis & Cowan, 1985), although the phosphate-rich COOH terminals of NF150 and NF200 have yet to be determined. Our ^{31}P NMR study of isolated NF150 and NF200 indicates that both polypeptides possess phosphate groupings in similar environments, since the chemical shifts of ^{31}P resonances from the respective polypeptides are very similar. NF150 and NF200, therefore, share some common structural features. On the other hand, the different resonance intensities observed for the homologous peaks indicate that the primary sequences in the phosphorylated regions of respective polypeptides are variable.

The definitive assignment of various ^{31}P signals and the significance of upfield vs. downfield resonances of NF proteins are not known at present. Nevertheless, some information concerning the locations and functions of different phosphate groups may be inferred from the use of selected biological probes. For example, of the three NF polypeptides, NF200 has been reported to promote nucleation of tubulin polymerization and to enhance microtubule assembly (Minami & Sakai, 1983). The perturbation of the ^{31}P NMR caused by microtubule proteins indicates that phosphate groups are very likely involved in such interactions. The data also point to certain NF phosphate groups, among others, that possess a higher affinity for microtubule proteins.

The perturbation studies with monoclonal antibody also provide information that could not be obtained by a standard immunochemical assay. These studies identify particular phosphate groups involved in the antibody binding as well as the relative proximity of the various phosphate groups with respect to each other. Moreover, the findings raise the possibility that ^{31}P NMR studies are able to provide a more detailed assignment of the phosphate-dependent epitopes recognized by monoclonal antibodies.

NF proteins are known to be cleaved by CANP in a stepwise fashion along their COOH-terminal segments (Eagles et al. (1981). ^{31}P NMR studies have shown that the phosphorylated regions of NF proteins are largely spared. Chymotrypsin, trypsin, and *Staphylococcus aureus* V8 protease also have been reported to leave phosphorylated regions intact (Julien & Mushynski, 1983). These findings make the isolation of phosphorylated segments of NF proteins and the quantitative determination of ^{31}P NMR parameters possible in the future.

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Registry No. Phosphoserine, 407-41-0; phosphothreonine, 1114-81-4.

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Detection of Energy Transfer between Tryptophan Residues in the Tubulin Molecule and Bound Bis(8-anilinonaphthalene-1-sulfonate), an Inhibitor of Microtubule Assembly, That Binds to a Flexible Region on Tubulin†

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ABSTRACT: The fluorescent apolar probe bis(8-anilinonaphthalene-1-sulfonate) (Bis-ANS) is a potent inhibitor of microtubule assembly that binds to tubulin at a hitherto uncharacterized site distinct from those of the antimitotic drugs. We have found that energy transfer between tryptophan residues and bound Bis-ANS leads to quenching of the intrinsic tubulin fluorescence. The quenching is biphasic, implying two types of Bis-ANS binding sites. The estimated K_d values are 2.7 and 22.2 μM, consistent with reported values for the primary and secondary Bis-ANS binding sites. Preincubation of tubulin at 37 °C results in increased quenching of tryptophan fluorescence without any effect on the K_d values, suggesting localized structural change in the protein around the Bis-ANS binding sites. Concentration-dependent depolarization of Bis-ANS fluorescence was observed, suggesting energy transfer among bound Bis-ANS molecules. Such a concentration-dependent decrease in fluorescence polarization was not observed with 8-anilinonaphthalene-1-sulfonate (1,8-ANS), the monomeric form of Bis-ANS. Perrin-Weber plots were obtained for bound Bis-ANS and 1,8-ANS by varying the viscosity with sucrose. The rotational relaxation times calculated for Bis-ANS and 1,8-ANS are 18 and 96 ns, respectively. Comparison with the theoretical value (125 ns) suggests that Bis-ANS binds to a flexible region of tubulin. This, coupled with the fact that Bis-ANS, but not 1,8-ANS, inhibits microtubule assembly, suggests that the region in the tubulin molecule responsible for microtubule assembly is relatively flexible.

The fluorescent apolar molecule bis(8-anilinonaphthalene-1-sulfonate) (Bis-ANS)[1] binds tightly to tubulin (K_d = 2 μM) and potently inhibits microtubule assembly (Horowitz et al., 1984). Because Bis-ANS is highly fluorescent, we have used it as a probe to learn about the region to which it binds, a portion of the tubulin molecule which is likely to be directly involved in microtubule assembly. The binding site of Bis-ANS on the tubulin molecule is unknown, but our previous work suggests that it does not overlap with the binding sites of colchicine, podophyllotoxin, vinblastine, or maytansine (Prasad et al., 1986). We report here the discovery of energy transfer between the tryptophan residues of tubulin and bound Bis-ANS and that the region to which Bis-ANS binds appears to be flexible.

Materials and Methods

Materials

Bis-ANS and 1,8-ANS were obtained from Molecular Probes Inc. (Junction City, OR). All other reagents were of analytical grade.

Methods

Tubulin Preparation. Bovine brain tubulin was purified by phosphocellulose chromatography as described earlier (Prasad et al., 1986).

Protein measurements were made according to the method of Lowry et al. (1951) using bovine serum albumin as standard.

Fluorescence Measurements. Fluorescence measurements were made with a Perkin-Elmer MPF-44A spectrofluorometer used in the ratio mode and equipped with temperature-controlled cell holder as described earlier (Prasad et al., 1986). The observed fluorescence intensities were corrected for the absorption of Bis-ANS using the relation (Lakowicz, 1983)

$$F_{cor} = F_{obsd} \text{ antilog } (OD_{ex} + OD_{em}/2)$$

where OD_{ex} and OD_{em} are the optical densities at the excitation and emission wavelengths, respectively.

Polarization Measurements. Fluorescence polarization measurements were made on an SLM Model 4800 spectrophotometer (SLM Instruments, Urbana, IL). The nature of

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[1] Abbreviations: Bis-ANS, bis(8-anilinonaphthalene-1-sulfonate); 1,8-ANS, 8-anilinonaphthalene-1-sulfonate; MAPs, microtubule-associated proteins.

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