Physicochemical Characterization of the 68 000-Dalton Protein of Bovine Neurofilaments†

Miriam R. Lifsics‡ and Robley C. Williams, Jr.*

ABSTRACT: The 68 000-dalton protein from bovine neurofilaments was purified by a combination of chromatography on DEAE-cellulose and on hydroxylapatite in buffers containing 8 M urea. Although the separation of this protein from the other proteins of the neurofilament appeared to be hampered by a mixed association of the several components, a nearly homogeneous product was obtained for study. Sedimentation equilibrium experiments in buffers containing 8 M urea showed the molecule to be a monomer with a molecular weight of 70 600 ± 2000. Circular dichroic spectra taken under the same conditions gave no evidence of residual α-helix. Molecular sieve chromatography in 8 M urea on controlled-pore glass showed that the molecule eluted at an unexpectedly small volume. The small elution volume did not depend significantly on protein concentration and is unlikely to be the result of intermolecular association. Rather, the monomer probably has a conformation more rigid or extended than a classical random coil. When dialyzed into 0.01 M tris(hydroxymethyl)aminomethane/1 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid/0.1 mM dithioerythritol, pH 8.5, the protein does not assemble into filaments. Sedimentation velocity reveals that under these conditions it consists mainly of a 4.8S molecular species, containing few large particles; sedimentation equilibrium shows that it is composed of oligomers, the smallest present in significant concentration having a molecular weight approximately that of a trimer. Circular dichroism measurements lead to the interpretation that the molecule has refolded in this buffer into a structure that has approximately 55% α-helix. Assembly into filamentous particles resembling neurofilaments occurs when the protein is dialyzed against 0.1 M 2-(*N*-morpholino)ethanesulfonic acid/0.1% β-mercaptoethanol/1 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid/0.17 M NaCl, pH 6.5. We suggest that the oligomeric species present in 0.01 M tris(hydroxymethyl)aminomethane may frequently be present in solubilized preparations of intermediate filaments and may represent an intermediate in the assembly process.

Neurofilaments, the intermediate filaments of mammalian neurons, are composed of three types of subunit polypeptides: one of 68 000 daltons (NF68),[1] one of 160 000 daltons (NF160), and one of 210 000 daltons (NF210) [for reviews, see Lazarides (1982) Schlaepfer (1979), and Williams & Runge (1983)]. Current evidence from antibody labeling (Willard & Simon, 1981; Sharp et al., 1982) and from reassembly studies (Geisler & Weber, 1981; Liem, 1982; Liem & Hutchison, 1982; Zackroff et al., 1982) suggests that the filament consists of an inner core of NF68, with NF160 and NF210 arrayed peripherally in a still unknown fashion. In common with other types of intermediate filaments, neurofilaments are quite stable in neutral buffers of moderate ionic strength (Schlaepfer, 1978), but they can be solubilized in denaturing solvents such as 8 M urea or 6 M guanidine hydrochloride. The subunits have been chromatographically resolved from each other (Willard et al., 1980; Moon et al., 1981; Geisler & Weber, 1981; Chiu & Norton, 1982; Hogue-Angelletti et al., 1982; Liem, 1982; Liem & Hutchison, 1982) in denaturing solvents, although such separations have evidently been difficult and have not been thoroughly documented. Solutions of purified NF68 will spontaneously reassemble into fairly uniform filaments of approximately 10-nm diameter when they are dialyzed into buffers of approximately 0.2 M ionic strength (Geisler & Weber, 1981; Liem & Hutchison, 1982; Zackroff et al., 1982). However, when NF160 or NR210 is added to the reconstitution mixture, shorter filaments are formed. Studies of amino acid sequences (Geisler et al., 1982; Steinert et al., 1983; Weber & Geisler, 1982) make a strong case for extensive homology between NF68 and several of the other intermediate filament subunits: desmin, vimentin, and several of the keratins. These data, taken together, point to NF68 as the central protein in the neurofilament, and the one through which neurofilament subunits may possess commonality of primary and secondary structure with subunits of other types of intermediate filaments.

The mechanism of reassembly of filaments from dissociated and unfolded NF68 and the structure of the filaments thus formed are unknown. Neurofilaments observed in partially denaturing buffers (Schlaepfer, 1977a,b) appear to consist of an unknown number of protofilaments, a structural feature that they share with other intermediate filaments. The number of NF68 subunits that are present in the cross section of a protofilament is also unknown ı the possibly analogous cases

† From the Department of Molecular Biology, Vanderbilt University, Nashville, Tennessee 37235. *Received October 17, 1983.* This investigation was supported by Grant GM 29834 from the National Institutes of Health and by a grant from the Vanderbilt University Research Council. M.R.L. was the recipient of the predoctoral award from Training Grant 732-GM07319 of the National Institutes of Health. This work was presented in partial fulfillment of the requirements for the Ph.D. degree at Vanderbilt University.

‡ Present address: Department of Anatomy, Vanderbilt University Medical School, Nashville, TN 37232.

[1] Abbreviations: NF68, NF160, and NF210, polypeptide subunits of bovine neurofilament of molecular weights 68 000, 160 000, and 210 000, respectively; CD, circular dichroism; DTE, 1,4-dithioerythritol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane; Cl_3CCOOH, trichloroacetic acid; EDTA, ethylenediaminetetraacetic acid; buffer A, 10 mM sodium phosphate, pH 7.5/1 mM EGTA/0.1% β-mercaptoethanol/8 M urea; buffer B, 10 mM sodium phosphate, pH 7.4/1% β-mercaptoethanol/20 mM ethanolamine/1 mM EGTA/8 M urea; buffer C, 10 mM Tris, pH 8.5/0.1 mM DTE/1 mM EGTA; buffer D, 150 mM sodium phosphate, pH 7.4/10 mM β-mercaptoethanol/20 mM ethanolamine/8 M urea; PM buffer, 0.1 M Pipes, pH 6.9/1 mM $MgSO_4$/2 mM EGTA/2 mM DTE.

0006-2960/84/0423-2866$01.50/0

of keratin, vimentin, and desmin, the subunit proteins are probably organized within the protofilament into coiled coils which are aligned with their axes roughly parallel to the filament axis (Renner et al., 1981; Wais-Steider et al., 1983), and the coiled-coil structure probably arises from lateral interactions between helical regions on neighboring molecules. The subunits of intermediate filaments of keratin, vimentin, desmin, and NF68 are thought to possess a homologous central region of α-helix, composing about 50% of their sequence (Geisler et al., 1982; Steinert et al., 1978, 1980, 1983; Renner et al., 1981; Wais-Steider et al., 1983). A major problem in reconstituting the filaments after separation of their proteins in a denaturing solvent is to assure that this element of secondary structure is properly regained.

This paper describes the separation and physical characterization of soluble NF68 from bovine spinal cord. It shows that, after purification in 8 M urea, the protein is present as monomeric polypeptide chains which contain no appreciable amount of α-helix. Upon removal of urea by dialysis into 10 mM Tris buffer of pH 8.5, the protein refolds into a highly α-helical species of $s_{20,w}$ approximately 4.8 S. This material does not form filaments under these conditions but is shown by equilibrium sedimentation to exist as a series of oligomers. No appreciable amount of monomer is present, and the smallest oligomer present in significant amounts appears to be a trimer. When this material is dialyzed into 10 mM Tris/0.17 M NaCl, it forms filaments that resemble those seen by other workers (Geisler & Weber, 1981; Moon et al., 1981; Liem & Hutchison, 1982; Zackroff et al., 1982).

Materials and Methods

Reagents. Pipes, EGTA, and DTE were obtained from Sigma Chemical Co.; ultrapure Tris, glycine, urea, and guanidine hydrochloride were obtained from Schwarz/Mann, Inc. Hydroxylapatite was Bio-Gel HTP, purchased from Bio-Rad Laboratories. Before use, solutions of urea were passed through a 3 × 40 cm column of Rexyn-300 (Fisher) to remove cyanate ions.

Preparation of Spinal Cord Neurofilaments. Bovine spinal cord neurofilaments were prepared by modifications of the method of Delacourte et al. (1980). Spinal cords were obtained from freshly slaughtered animals (Elm Hill Meats, Nashville, TN), stored on ice, and processed within 1 h after slaughter. All operations were performed at 4 °C except where noted. Minced tissue (75-g aliquots) was homogenized into 100 mL of PM buffer in a Sorvall Omni-mixer for 50 s at speed 3 and for 10 s at speed 9. The homogenate was centrifuged at 6000g for 15 min in a Sorvall GSA rotor. The supernatant was recovered and further centrifuged at 95000g for 75 min in a Beckman type 35 rotor to remove particulate matter. The supernatant was made 20% (v/v) glycerol, warmed to 37 °C for 20 min, and centrifuged at 95000g for 60 min at 37 °C. The glycerol supernatant was decanted and saved at 4 °C. The pellet was resuspended in 60 mL of PM buffer by homogenization in a motor-driven Potter homogenizer with a Teflon pestle at speed 4 for 1 min at 4 °C and was then centrifuged at 95000g for 60 min at 4 °C. The supernatant from this centrifugation was made 20% glycerol (v/v) and combined with the glycerol supernatant from the previous centrifugation. The pellet was resuspended in 10 mL of PM buffer by homogenization and was termed first cycle filaments. The warming–centrifugation cycle was repeated on the pooled supernatant from the two previous centrifugations. This pellet was resuspended as described and termed second cycle neurofilaments. Both preparations were frozen dropwise in liquid nitrogen and stored at −80 °C.

Polyacrylamide Gel Electrophoresis. Discontinuous sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed on 8% slab gels with 3% stacking gels according to the method of Laemmli (1970). Ultrasensitive silver staining was done by the methods of Oakley et al. (1980) and Wray et al. (1981). Slab gels (0.75 mm thick) were soaked overnight in 50% methanol, fixed for 30 min in 10% glutaraldehyde, and washed for a minimum of 2 h in distilled, deionized water. Gels were stained as described by Wray et al. (1981). Artifactual bands of stained material that appear at positions on the gel corresponding to 55 000 and 67 000 daltons were seen in silver-stained gels (Tasheva & Desser, 1983). These bands appear to arise from components of the sample buffer (Tasheva & Desser, 1983). They were reduced in intensity but not eliminated by the use of freshly prepared sample buffer or by filtration of the sample buffer (M. R. Lifsics, unpublished results).

Purification of Triplet Proteins. Triplet polypeptides were purified, by chromatography on DEAE-cellulose and on hydroxylapatite in buffers containing 8 M urea, by modifications of the methods of Geisler & Weber (1981) and of Liem (1982). Neurofilaments (80–100 mg) were solubilized in 10 mL of buffer A (10 mM sodium phosphate, pH 7.5, 1 mM EGTA, 0.1% β-mercaptoethanol, and 8 M urea) and dialyzed against 100 mL of this buffer for 12 h at room temperature. The resulting solution was centrifuged at 95000g for 60 min at 22 °C to pellet aggregated material. The protein concentration of the supernatant was adjusted to 2 mg/mL, and the solution was applied to a 2 × 16 cm DEAE-cellulose (DE-52, Whatman) column equilibrated with buffer A. Absorbed protein was eluted by means of a linear 0–0.250 M NaCl gradient of 2000-mL total volume. A constant flow rate of 40 mL/(h·cm^2) was maintained with a Buchler Dekastaltic pump. Protein concentration of individual fractions (8 mL) was assayed by the method of Bradford (1976). Protein composition was analyzed by SDS–PAGE (Laemmli, 1970).

Further purification of each of the individual triplet proteins was obtained by chromatography on hydroxylapatite in 8 M urea. Fractions from DEAE chromatography enriched in a particular protein were pooled and dialyzed against 10 mM sodium phosphate buffer, 1 mM EDTA, and 0.1% β-mercaptoethanol for 24 h at 4 °C. Protein was precipitated by addition of 20% Cl_3CCOOH and pelleted by centrifugation for 20 min at 4 °C. Pellets were resuspended in 6 M guanidine hydrochloride, 1 mM EGTA, and 1% β-mercaptoethanol and dialyzed into buffer B (10 mM phosphate, pH 7.4, 1% β-mercaptoethanol, 20 mM ethanolamine, 1 mM EGTA, and 8 M urea). Protein concentration was adjusted to less than 5 mg/mL, and the solution was applied to a 0.9 × 10 cm column equilibrated with buffer B. The column was then washed with buffer B that had been brought to 85 mM phosphate. Elution of absorbed protein was carried out by means of a linear phosphate gradient from 85 to 150 mM phosphate of total volume 100 mL. A flow rate of 0.11 mL/(h·cm^2) was maintained with a Buchler Dekastaltic pump. Fractions were collected in 1-mL aliquots and assayed as described. The hydroxylapatite–DEAE-purified protein was dialyzed into 10 mM Tris, pH 8.5, 1.0 mM EGTA, and 1 mM DTE, frozen dropwise in liquid nitrogen, and stored at −80 °C.

Ultracentrifugal determinations of the molecular weight of purified 68K protein in the presence of a denaturant were performed in buffer D (150 mM phosphate, pH 7.4, 1% β-mercaptoethanol, 20 mM ethanolamine, and 8 M urea). Protein was dialyzed directly into this buffer for 48 h after

hydroxyapatite chromatography or after prior storage at −80 °C in 10 mM Tris buffer. Equilibration of buffer components was monitored by inclusion of a dialysis bag containing only the original buffer. At intervals, aliquots were removed from this bag, and their refractive index, pH, conductivity, and ultraviolet absorption were measured. All four properties were found to reach equilibrium, within experimental uncertainty, by 36 h of dialysis.

Molecular weight determinations in the absence of a denaturant were made in 10 mM Tris, pH 8.5, 0.1 mM DTE, and 1 mM EGTA (buffer C). Protein was dialyzed against 4000 volumes of this buffer for 48 at 2 °C. An 8 M urea buffer blank was included during dialysis and was checked for equilibrium against Tris buffer by measurement of pH, conductivity, refractive index, and UV absorption. After dialysis, protein solutions were centrifuged at 180000*g* for 60 min at 4 °C in a Beckman type 40 rotor to remove insoluble aggregates.

Absorption Spectra. Absorption measurements were performed in a Cary 118C spectrophotometer at 4 °C to determine the wavelength of maximum absorbance for use with the photoelectric scanner of the ultracentrifuge and for determination of an extinction coefficient. Concentrations were estimated from refractive indices measured in the analytical ultracentrifuge according to Babul & Stellwagen (1969). A specific refractive index increment of 2.206×10^{-3} dL/g (for a 12-mm optical path at 632.8 nm) was employed (Detrich, 1979).

Protein Determination. Standard curves for the Bradford (1976) and Lowry (1951) assays were prepared by the use of dilutions of the same solution used to measure the extinction coefficient.

Circular Dichroism Spectroscopy. CD spectroscopy was performed in 10 mM Tris buffer, pH 8.4. CD spectra were obtained by the use of a Cary 60 spectropolarimeter. All solutions were scanned 2 or 3 times at 23 °C over a wavelength range of 190–260 nm. Full-scale sensitivity was set at 4.0×10^{-2} deg and the time constant at 3 s. Path lengths were 2 and 0.5 mm for solutions in 8 M urea and 10 mM Tris, respectively. Protein concentrations were between 0.1 and 0.2 mg/mL. Mean residue weights, calculated from the amino acid composition determined by Hogue-Angeletti et al. (1982), were 138 for NF68, 126 for NF160, and 125 for NF210. Curve resolution and data analysis were performed by J. O. Gailit by means of a program that searches for percentages of α-helix, β-structure, β-turns, and aperiodic structure from reference spectra of Chang et al. (1978) (15 proteins of known crystallographic structure). The program does not force constraints either on the fractions of the individual components (between zero and one) or on their sums (to unity). The fraction of α-helix was calculated according to Greenfield & Fasman (1969) from the value of the circular dichroism at 208 nm.

Molecular Sieve Chromatography. Molecular sieve chromatography was performed on glycerol-coated, controlled-pore glass beads (Electro-Nucleonics) of 50-nm pore size in a 0.9 × 90 cm column. The column was equilibrated with buffer D (150 mM sodium phosphate, pH 7.4, 10 mM β-mercaptoethanol, 20 mM ethanolamine, and 8 M urea). Void and internal volumes were calibrated with salmon sperm DNA and 0.5 M sodium phosphate, respectively. Experimentally determined void and internal volumes agreed to within 5% with values calculated from the manufacturer's specifications. Molecular weight markers were thyroglobulin (subunit molecular weight of 350 000), conalbumin (77 000) (Timasheff & Tinoco, 1957), catalase (63 000), ovalbumin (43 000), and chymotrypsinogen A (25 000). Conalbumin was purchased from Sigma Biochemicals. The other proteins was obtained from Pharmacia Fine Chemicals, and the molecular weights specified by the manufacturer were used. Gel filtration of NF68 was performed at five different initial concentrations (between 0.290 and 1.660 mg/mL) in identical loading volumes (0.5 mL) to determine the concentration dependence of the elution volume. Eluant volumes were obtained by collecting fractions in tared test tubes which were subsequently weighed to 0.01 g.

Analytical Ultracentrifugation. Sedimentation experiments were performed in a Beckman Model E analytical ultracentrifuge equipped with a photoelectric optical absorption scanner and a modulated laser light source (Williams, 1972). Sedimentation equilibrium experiments were performed in cells having three double sectors, each 3 mm long in the radial dimension (Yphantis, 1964), while sedimentation velocity experiments were performed in standard double-sector cells. When the Rayleigh interference optical system was employed to measure concentration distributions, fringe patterns were recorded on Kodak 2415 film, and fringe displacements were measured by means of a computer-equipped Nikon comparator.

Sedimentation in 8 M urea was observed by means of the absorption scanner. Data were corrected for the estimated binding of 1 mol of urea per peptide bond plus 1 mol of urea per aromatic residue (Prakash et al., 1981). A value of 324 mol of urea/mol of protein was estimated from the amino acid composition of the 68K protein (Hogue-Angeletti et al., 1982). Solution density was obtained by pycnometry. Partial specific volume was calculated according to the method of Cohn & Edsel (1943) from the amino acid composition determined by Hogue-Angeletti et al (1982). Centrifugation in 10 mM Tris buffer was recorded by a Rayleigh interference optical system. Local apparent weight-average molecular weights, $M_{w,app}$, were calculated from blank-corrected data by means of a fitting program similar to those described by Roark & Yphantis (1969) and by Teller (1973).

Model Calculations. Simulated curves of protein distribution at equilibrium for an ideal two-component system that contains n species of noninterconverting protein oligomers were generated from

$$C_i(r) = C_{i,o} \exp[\sigma_i(r^2 - a^2)/2]$$

$$C(r) = \sum_{i=1}^{n} C_i(r)$$

Here, $C_i(r)$ is the concentration of the ith species at radius r, $C_{i,o}$ is its concentration at the meniscus of the assumed solution column, and σ_i is its reduced molecular weight (Yphantis, 1964). $C(r)$ is the total concentration at radius r, and a is the radius of the assumed meniscus. Rotor speeds, total initial concentrations, and radii of menisci were chosen to be equal to those in the given real experiment being simulated.

Reassembly Experiments. In vitro reassembly of the 68K protein was performed according to the method of Geisler & Weber (1981). NF68 at 0.5 mg/mL was dialyzed against 500 mL of reassembly buffer (0.1 M Mes, 0.1% β-mercaptoethanol, 1 mM EGTA, and 0.17 M NaCl) at 37 °C. Aliquots were removed at various time intervals, and the degree of reassembly was assayed by observation of negatively stained preparations in the electron microscope.

Electron Microscopy. Negative staining was performed by application of 10-μL aliquots of solution to 400-mesh Form-

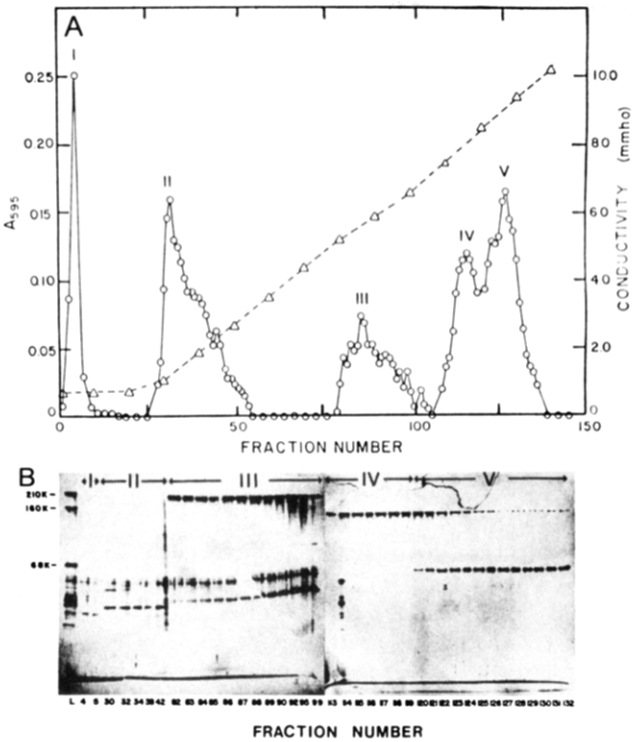

FIGURE 1: Chromatography and electrophoresis of neurofilament preparations on DEAE-cellulose. (A) Forty to eighty milligrams of first cycle neurofilament protein was solubilized in buffer A (10 mM phosphate, 0.1% β-mercaptoethanol, 1 mM EGTA, and 8 M urea) and applied to a 2 × 16 cm column of DE-52 (Whatman). Protein was eluted with a linear 0–0.250 M NaCl gradient of 2000-mL total volume. A flow rate of 40 mL/(h·cm^2) was maintained with a Buchler Dekastaltic pump. A_{595} as measured in the Bradford assay (O); conductivity (mmho) (Δ). (B) Electrophoresis of fractions obtained from DEAE chromatography described in (A). Roman numerals denote positions of fractions corresponding to peaks.

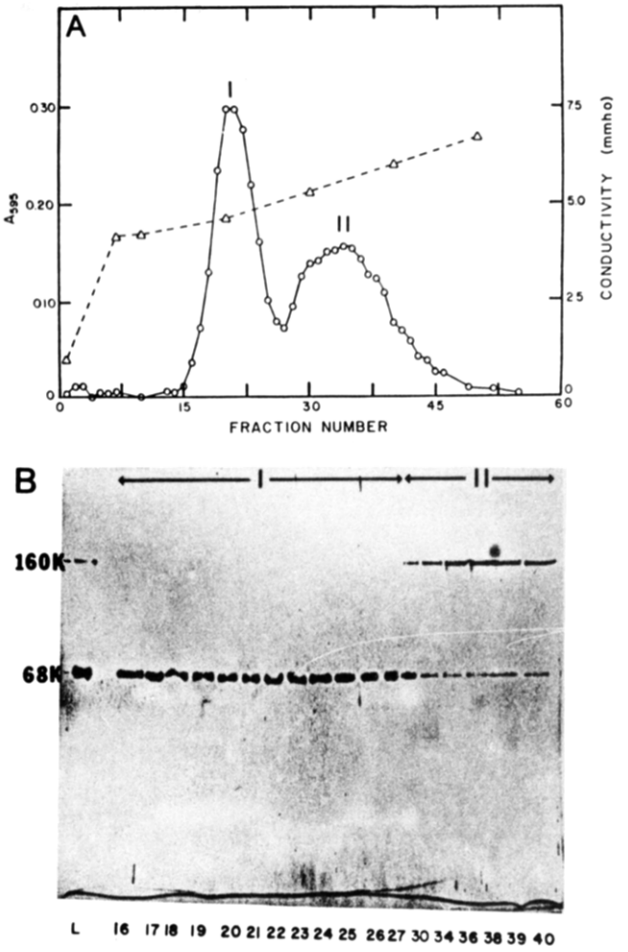

FIGURE 2: Hydroxylapatite chromatography and electrophoresis of NF68-enriched fractions obtained from DEAE chromatography. (A) The pooled fractions were dialyzed into 10 mM phosphate buffer, pH 7.5, 2 mM DTE, and 1 mM EGTA, precipitated by 20% Cl_3C-COOH, resuspended into 6 M guanidine hydrochloride, and dialyzed into buffer B (10 mM phosphate, 1% β-mercaptoethanol, 20 mM ethanolamine, 1 mM EGTA, and 8 M urea). The protein concentration was adjusted to below 5 mg/mL prior to being loaded on a 0.9 × 10 cm hydroxylapatite column prepared as described under Materials and Methods. Protein was eluted with a linear 85–150 mM phosphate gradient of 100-mL total volume. A flow rate of 0.11 mL/(hr·cm^2) was maintained with a Buchler Dekastaltic pump. A_{595} as detected in the Bradford assay (O); conductivity (mmho) (Δ). (B) Electrophoresis of fractions shown in (A). Roman numerals denote approximate locations of peaks.

var-coated grids covered with approximately 20 Å of carbon. Grids were glowed immediately before use. The solution was allowed to adhere to the grids for 10 s and was then stained with 10 drops of 2% uranyl acetate. Excess stain was absorbed with filter paper, and the grids were air-dried prior to examination in a Jeol 100-S electron microscope operated at 80 kV.

Results

Purification of Individual Triplet Polypeptides. Figure 1A shows the elution profile obtained by DEAE chromatography of neurofilament protein in 8 M urea. The protein compositions of selected fractions are shown in Figure 1B. No triplet proteins of the neurofilament triplet NF68, NF160, and NF210 were observed in the eluant at NaCl concentrations below 85 mM (conductivity below 4.0 mmho). The dominant band in peak I was a 43 000-dalton protein (possibly actin) which was not further characterized. The major component in peak II was a 50 000-dalton protein which has been identified as the glial filament subunit polypeptide by Liem (1982). The triplet proteins and a 55 000-dalton polypeptide were eluted between 85 and 250 mM NaCl. The 210K polypeptide was found primarily in peak III, accompanied by a trailing shoulder containing several bands that migrated slightly faster than the 210K polypeptide on SDS–PAGE. These may be degradation products of the 210K protein. A 55 000-dalton protein, possibly tubulin, was also observed in the shoulder on the trailing edge.

Peak IV enriched in the 160K subunit while peak V was enriched in the 68K subunit. Attempts to increase resolution of the 160K and 68K proteins by changing the elution gradient to 85–200 mM salt, by increasing the length of the column, by decreasing loading concentrations to 1 mg/mL, or by decreasing the amount of protein applied to 40 mg had no significant effect. An 85–250 mM gradient increased resolution of the 210K polypeptide from the two other triplet proteins and was used in some preparations.

Further purification of the NF68-enriched fractions in peak V was achieved by chromatography on hydroxylapatite. An elution profile is shown in Figure 2A. Fractions eluting at the leading edge of peak I contained highly purified 68K protein (Figure 2B). This material was pooled and frozen for the characterization described below.

Extinction Coefficient. An absorption maximum in 0.01 M Tris, pH 8.5, was observed at 278.5 nm. A value of 0.374 ± 0.04 mL mg^{-1} cm^{-1} was obtained for the extinction coefficient of NF68 in buffer. This small value likely reflects the paucity of aromatic residues in NF68 (Hogue-Angeletti et al., 1982).

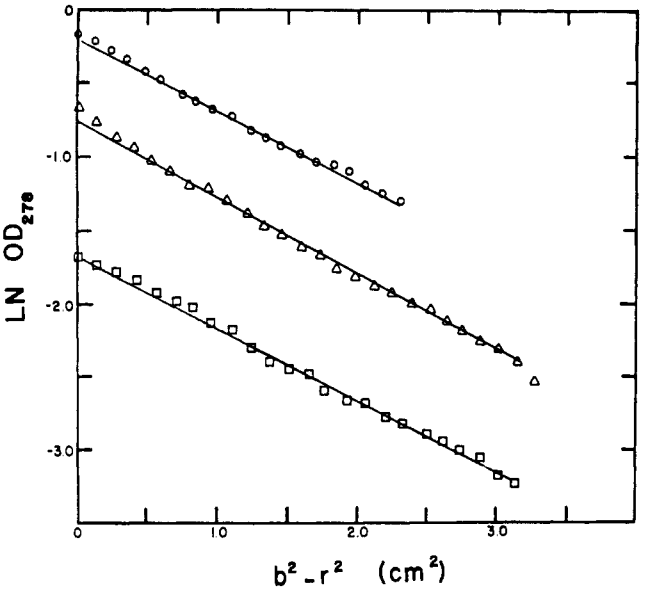

FIGURE 3: Equilibrium sedimentation of purified NF68 in 8 M urea. The horizontal axis is the radius of the cell base squared minus the radius squared. Initial concentrations (all from a single preparation) were 1.25 (○), 0.6 (△), and 0.32 mg/mL (□). Rotor speed was 10000 rpm, and scans were taken 36 h after the start of the experiment. Temperature, 17 °C; wavelength of light, 278.5 nm.

Characterization of NF68 in 8 M Urea. Previous results from both molecular sieve and ion-exchange chromatography experiments indicated that NF68 formed mixed aggregates with the 160K and 210K proteins in 8 M urea (M. R. Lifsics, unpublished results). To determine whether the purified NF68 also self-associates in 8 M urea, the molecular weight of the protein was obtained by equilibrium sedimentation in 8 M urea.

Equilibrium sedimentation was performed at three different initial concentrations. Figure 3 shows plots of the natural logarithm of optical density as a function of $b^2 - r^2$, where b is the radius of the base of the cell and r is any other radius. In channels A and C, the logarithm of optical density increased linearly with increasing radial distance. Channel B exhibited small but significant curvature, which may arise from the presence of a small fraction of aggregates. Slopes were determined from lines fitted by least-squares analysis. $M_{w,app}$, corrected for bound urea, for channels A, B, and C was 68000, 74000, and 70000, respectively. The near-equality of the slopes of these lines indicates (Yphantis, 1964) that the macromolecular solute is homogeneous within the limits of detection of the method. The close agreement between the present results and those obtained by SDS–gel electrophoresis makes it likely that NF68 is a protein of molecular weight near 70000 and that it exists as a monomer in 8 M urea.

The Stokes radius of NF68 protein was estimated by molecular sieve chromatography in 8 M urea on a controlled-pore glass column. The column was calibrated in 8 M urea with five proteins that had elution positions that were a logarithmic function of their respective molecular weights. The elution volume of NF68 was considerably smaller than would be predicted from the calibration curve, corresponding to an apparent molecular weight of 116300 ± 1500. The range of elution volumes observed over a 4-fold range of concentration (0.290–1.660 mg/mL) corresponded to molecular weights between 100000 and 130000. Elution volume was not correlated with concentration, indicating that formation of reversibly associated aggregates is unlikely to be responsible for the observed large elution volume. Likewise, the presence of irreversibly associated aggregates is also unlikely, since they would have been easily seen in the sedimentation equilibrium experiments.

Table I: Estimation of Secondary Structure of NF68 in 10 mM Tris Buffer[a]

subunit	f_α[b]	f_β[c]	f_t[d]	f_a[e]	f_Σ[f]
68K	0.53	0.54	−0.21	0.12	0.98
160K	0.11	0.58	−0.05	0.40	1.04
210K	−0.06	1.58	−0.05	1.07	2.54

[a] Individual transitions were estimated from nonconstrained linear fitting to reference spectra of 15 proteins of known crystallographic structure (Chang et al., 1978). [b] Fraction of residues in α-helix. [c] Fraction of residues in β-sheet. [d] Fraction of residues in β-turn. [e] Fraction of residues in aperiodic form. [f] Sum of all components.

The lack of detectable heterogeneity in the sedimentation equilibrium experiments suggests that the anomalous elution position may arise from conformational effects. CD spectroscopy was performed on the 68K protein in 8 M urea to determine the presence of residual secondary structure. Mean residue ellipticity at 222 nm was −2143 ± 887 deg cm^2 dmol^{-1}, indicating that appreciable α-helical domains are absent. Additional secondary structure (β-sheet or β-turns) could not be ruled out since scanning in the far UV could not be performed due to high solvent absorbance at wavelengths below 220 nm. The data suggest that the protein is a monomer that retains some secondary or tertiary structure that is not detectable by CD.

In contrast to the chromatographic behavior of NF68, the 160K protein elutes with a K_{av} corresponding to a molecular weight of 169000, in good agreement with the value obtained by SDS–PAGE.

Characterization of NF68 in 10 mM Tris Buffer. Changes in secondary structure and protein interactions were analyzed upon removal of urea to determine whether NF68 was capable of forming a soluble refolded species. Purified NF68 was dialyzed against 10 mM Tris buffer, pH 8.5, for 48 h at 2 °C. Solutions were clarified by centrifugation at 180000g for 60 min prior to use. Maximum protein concentration recovered in the supernatant was 1.6 mg/mL, a value similar to that obtained for purified desmin, which is soluble in 10 mM Tris, pH 8.5, to a concentration of 2.0 mg/mL (Huiatt et al., 1982). Large aggregates or intact filaments were absent in negatively stained preparations of these solutions. Approximately 75–90% of the NF68 remained in the supernatant of the 180000g × 60-min centrifugation at initial concentrations below 1.6 mg/mL. Hence, this concentration probably represents the maximum concentration at which these filaments are soluble.

CD spectroscopy was performed to determine the presence of secondary structure in NF68 in 10 mM Tris buffer. The CD spectrum is shown in Figure 4A. The mean residue ellipticity at 222.5 nm was 18793 deg cm^2 dmol^{-1}, a value indicative of high α-helical content. Individual $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ transitions were resolved by carrying out a nonconstrained fit to linear combinations of reference spectra of 15 proteins of known crystallographic structure (Chang et al., 1978). Fractions of α-helix, β-sheet, β-turns, and aperiodic structure thus obtained are shown in Table I. NF68 appears to be organized into roughly equal fractions of α-helix and β-sheet. An α-helical content of 58% determined at 208 nm by the method of Greenfield & Fasman (1969) is in good agreement with the 53% obtained from the curve-fitting program. Estimation of β-turns is extremely imprecise due to the many variants of the β-turn which yield wavelength differences up to several nanometers as well as variations in the magnitudes of individual bands (Chang et al., 1978). The

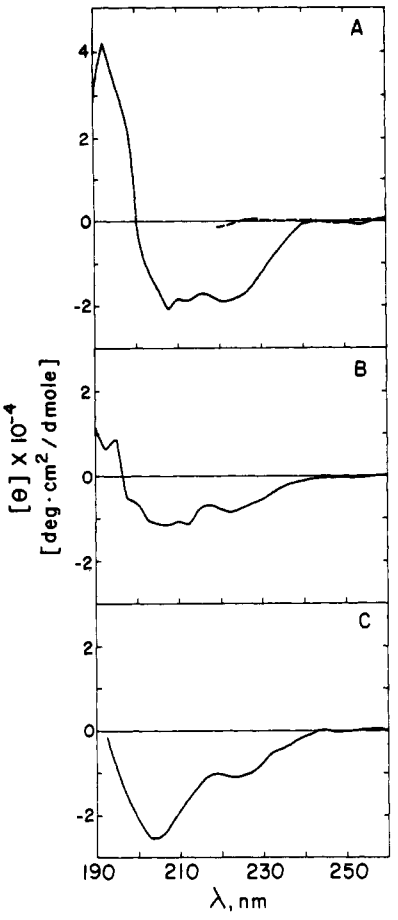

FIGURE 4: CD spectra of (A) NF68 at 0.125 mg/mL in 10 mM Tris buffer, pH 8.5 (solid line), and at 0.148 mg/mL in 8 M urea (dashed line). Measurements were performed as described under Materials and Methods. Protein concentration, determined by the Lowry assay, was 0.125 mg/mL in 10 mM Tris and 0.148 mg/mL in 8 M urea. (B) CD spectrum of purified NF160 at 0.120 mg/mL in 10 mM Tris. (C) CD spectrum of NF210 at 0.164 mg/mL in 10 mM Tris.

negative β-turn content reported here may result from variant forms of the β-turn not represented in the reference spectra.

The CD spectrum of NF68 is quite distinct from spectra of the 160K and 210K proteins (panels B and C, respectively, of Figure 4). NF160 has a high content of β-structure, approximately 58%, while the remainder of the molecule appears to be aperiodic. The spectrum of the 210K protein fits poorly any linear combination of the 15 reference spectra. The most likely ordered secondary structure appears to resemble β-sheet.

These results suggest that NF68 spontaneously folds into an ordered, highly α-helical structure in 10 mM Tris buffer upon removal of denaturant by dialysis. Its secondary structure is distinct from those of NF160 and NF210 under the same conditions. The secondary structures, as defined by CD spectroscopy, may not represent native structures.

Velocity sedimentation of NF68 at 0.233 mg/mL in 10 mM Tris yielded scans exhibiting a single apparent boundary and a flat plateau. While these findings are insufficient as criteria for homogeneity, they do indicate that large aggregates, if present, are at levels below 10% of total protein concentration. A sedimentation coefficient, $s_{20,w}$, of 4.8 S was obtained under these conditions. At concentrations of 1.6 mg/mL, a flat plateau was not obtained, a result indicative of the presence of self-association.

Results of sedimentation equilibrium experiments with NF68 in 10 mM Tris are shown in Figure 5. The values of molecular weight are based on a calculated value for $\bar{v}$ of 0.733 $cm^3\ g^{-1}$. Two experiments, comprising six different initial

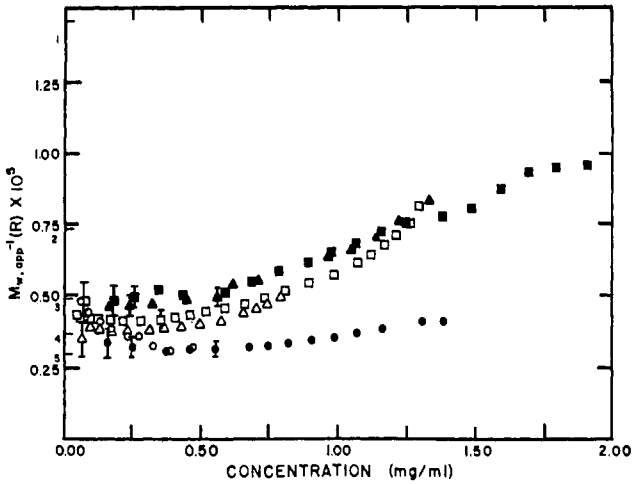

FIGURE 5: Reciprocal weight-average molecular weights of NF68 as a function of its concentration in 10 mM Tris, pH 8.5, 1 mM EGTA, and 0.1 mM DTE. Experiments were performed at 4 °C at a rotor speed of 14 000 rpm. Details of the experiments and analysis are described under Materials and Methods. Experiment I and experiment II were carried out with different preparations. Initial concentrations were as follows: experiment I, 0.100 (○), 0.150 (△), and 0.233 mg/mL (□); experiment II, 0.400 (●), 0.808 (▲), and 1.600 mg/mL (■). Small numbers on the vertical axis indicate multiples of 68 000 molecular weight. Error bars represent the estimated experimental uncertainty from all sources.

concentrations and protein from two different preparations, are represented. Reciprocal local weight-average apparent molecular weights, $M_{w,app}^{-1}$, are plotted as a function of concentration. Protein used in experiment I was frozen at −80 °C prior to use, while protein used in experiment II was used immediately after purification and was not subjected to freezing or storage. Several points can be made about the state of NF68 in 10 mM Tris. First, the nonoverlap of curves resulting from measurements at different initial concentrations indicates (Yphantis, 1964; Teller, 1973; Yphantis et al., 1978) that the solute must contain some noninteracting species (most probably oligomers) of different molecular weights. Second, the general upward trend of $M_{w,app}^{-1}$ with increasing concentration reflects positive nonideality, most likely resulting from a large contribution of the Donnan effect (Roark & Yphantis, 1971). Despite these two limitations, however, one can see that an extrapolation of the data to zero solute concentration suggests that the smallest detectable molecular species present has a molecular weight equal to or somewhat greater than 210 000 and thus would represent a trimer of NF68.

For closer examination of the possible origin of this heterogeneity, model calculations were performed in an attempt to generate the observed data at small concentrations from various reasonable combinations of noninteracting oligomers of NF68. Hypothetical mixtures containing only two species were chosen for simplicity. Simulated ideal data were compared to experimental results at concentrations below 0.2 mg/mL, where the contribution of nonideality to $M_{w,app}$ was estimated to be below 15%. The second virial coefficient was estimated, according to a standard expression (Roark & Yphantis, 1971), as being composed of a term due to the Donnan effect and one due to excluded volume. Neither of the required quantities, the electrostatic charge or the shape of the molecule, was directly available. The charge was estimated from the amino acid composition (Hogue-Angeletti, 1982), and the molecule was assumed to be a rod with an axial ratio of 50 (Gleisler & Weber, 1982; Steinert et al., 1980). Both values were chosen to give a maximum reasonable estimate of the second virial coefficient.

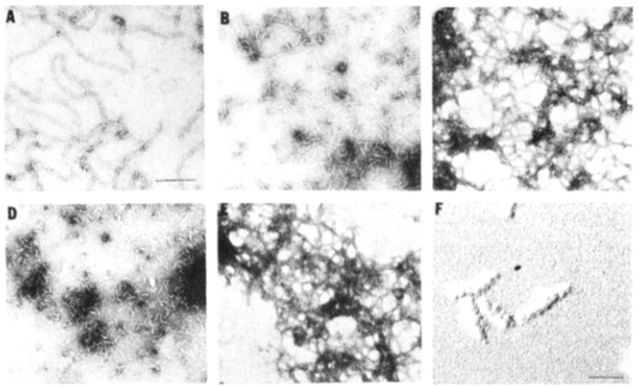

FIGURE 6: Electron microscopy of negative-stained preparations of reassembled filaments of NF68. Reassembly experiments were performed as described under Materials and Methods. (A) Second cycle spinal cord neurofilaments. (B) NF68 neurofilaments reassembled from 6 M guanidine hydrochloride. (C) NF68 filaments reassembled from 8 M urea. (D) NF68 filaments reassembled from 10 mM Tris, pH 8.5. (E) NF68 reassembled from 10 mM Tris, pH 8.5, for 8 h. (F) Metal-shadowed NF68 filaments in 10 mM Tris + 20% glycerol (panel F was generously provided by H. Erickson and L. Milam).

The best fits of calculated curves to the observed data of molecular weight vs. concentration were obtained for mixtures of trimer (210 000 daltons) with small amounts (~5–15%) of oligomeric species of somewhat higher molecular weight: tetramer, pentamer, or hexamer. Markedly poorer fits were obtained for mixtures of trimer with smaller oligomers (dimer or monomer), and the standard deviation of the fit had its smallest values when the proportions of dimer or monomer in those mixtures were equal to zero, indicating that no amount of material smaller than trimer needed to be invoked to explain the observed data. One can conclude that NF68, under these buffer conditions and at concentrations below 0.2 mg/mL, is not present as monomer or dimer in significant amounts. A trimer appears to be the smallest significant molecular species. Because of the heterogeneity and nonideality, nothing can be said about the presence of larger aggregates.

Assembly of Filaments of NF68 in Vitro. In vitro reassembly experiments were performed to determine if the oligomers of NF68 formed in 10 mM Tris were capable of polymerizing into 10-nm filaments, as does NF68 dialyzed from urea (Liem, 1982), guanidine hydrochloride (Geisler & Weber, 1981), or SDS (Zackroff et al., 1982) into near-neutral buffers at salt concentrations of 0.1 M.

NF68 was dialyzed at 37 °C from 10 mM Tris buffer, pH 8.5, into 100 mM Mes buffer, pH 6.5, containing 0.17 M NaCl (Geisler & Weber, 1981). Assembly products were analyzed after 3 h by electron microscopic examination of negatively stained preparations. These products were compared both to native neurofilaments and to products obtained by in vitro reassembly of NF68 dialyzed directly from 6 M guanidine hydrochloride or from 8 M urea into Mes reassembly buffer.

Results are shown in Figure 6. When dialyzed directly from 6 M guanidine, NF68 forms filaments 7–15 nm in diameter with lengths between 0.1 and 1.0 μm. Thin filaments, 3–7 nm in diameter, are also commonly observed. The primary reassembly products obtained from NF68 after dialysis from 8 M urea were large bundles of filaments. Individual filaments extending from the aggregates were 12–20 nm in diameter.

In contrast, the oligomers from 10 mM Tris buffer reassembled into short rods, less than 100 nm in length, with diameters ranging from 5 to 20 nm. Increasing dialysis times to 8 h resulted in the formation of large fibrous bundles similar to the aggregates formed after denaturation in urea. Metal-shadowed preparations of NF68 in 10 mM Tris buffer + 20% glycerol (v/v) contain short rods similar to the rods formed in Mes reassembly buffer (Figure 6). The shadowed preparations exhibited the 20-nm periodicity observed in native intermediate filaments (Milam & Erickson, 1982; Henderson et al., 1982).

Discussion

Separation of Neurofilament Proteins. Several laboratories have reported separations of proteins of the neurofilament triplet (Geisler & Weber, 1981; Moon et al., 1981; Liem, 1982; Liem & Hutchison, 1982; Willard et al., 1980; Hogue-Angelletti, 1981; Chiu & Norton, 1982), but none has provided a thorough discussion of the process. Column elution profiles have been published in only one of these reports (Chiu & Norton, 1982).

In the study reported here, chromatography of the triplet proteins on DEAE-cellulose and on HTP in 8 M urea yielded complex elution patterns. DEAE chromatography, performed according to Geisler and Weber, separated the NF210 protein from the others but separated NF68 from NF160 only poorly. Other authors (Chiu & Norton, 1982; Delacourte et al., 1982) have reported similar difficulties. The poor resolution of NF68 and NF160 may arise in part from a similarity of electrostatic charge. In addition, the proteins may tend to form associated complexes with each other, even in denaturing buffers. Qualitative data (M. R. Lifsics, unpublished results) from small-zone molecular sieve chromatography in 8 M urea show that elution position is a function of concentration in mixtures of the three neurofilament proteins, as one would expect if association were taking place. The presence of such association would impede separation. Another possible source of poor separation may arise from a broadening of the zones of elution of NF160 and NF210 due to heterogeneity in their extents of phosphorylation. Each of these proteins is extensively phosphorylated (Jones & Williams, 1982; Julien & Mushynski, 1982), and partial dephosphorylation of either could produce variations in electrostatic charge of more than 20 electronic charges near neutral pH. A near-continuum of phosphorylated forms of the same polypeptide chain would thus be expected to elute over a broad range of ionic strength and could easily produce the observed results.

Despite these difficulties, the combination of chromatography on DEAE-cellulose and hydroxylapatite provides a practical means of preparing milligram amounts of highly purified NF68 protein, in confirmation of the results of Geisler & Weber (1981) and of Liem & Hutchison (1982).

Characterization of NF68 in 8 M Urea. A molecular weight of 70 600 ± 2000 obtained from equilibrium sedimentation experiments shows that the purified NF68 exists as a single polypeptide chain in 8 M urea. The value obtained by equilibrium sedimentation may be systematically low due to possible overestimation of bound urea. Following Prakash et al. (1981), we assumed that the polypeptide chain is a random coil, each of whose binding sites has equal access to solvent, and thus estimated that one urea molecule is bound per peptide bond and one per aromatic residue. However, the results of molecular sieve chromatography indicate that NF68 does not behave as a random coil, because its elution volume in 8 M urea is considerably smaller than that predicted by the calibration curve for the CPG-500 column employed in this study. Hence, the molecule may have less urea bound than we estimate.

The large discrepancy between molecular weights obtained by molecular sieve chromatography and sedimentation equilibrium is of unknown origin. The small elution volume ob-

tained in chromatography may arise from conformational effects. Geisler & Weber (1980) have reported an anomalously small elution volume for purified desmin in 6 M urea and have attributed it to a lack of full denaturation of the molecule. In addition, paramyosin, a highly α-helical protein like NF68, exhibits elution behavior similar to that of NF68 in 8 M urea (Blagrove & Frenkel, 1977). Optical rotary dispersion measurements indicate that considerable α-helical structure is present in paramyosin in 8.6 M urea (Woods, 1969). However, the mean residue ellipticity value of the 68K protein at 222 nm, -2134 deg cm^2 dmol^{-1}, indicated that extensive domains of α-helix were absent. The presence of additional secondary structure could not be evaluated. Viscosity experiments should be able to provide more information on the conformation of NF68, but such experiments were not attempted since the amount of protein required was considerably greater than that available.

Characterization of NF68 in 10 mM Tris. The CD spectrum of NF68 in 10 mM Tris indicates that the protein spontaneously refolds into an ordered conformation in this buffer. Most residues are organized into regions of secondary structure consisting of α-helix or β-sheet. The amount of α-helix, approximately 53%, is in good agreement with the value of 58% determined by the method of Greenfield & Fasman (1969) at 208 nm. This value is less than values reported for NF68 from ox and cat which were approximately 70% after renaturation from urea solutions (Wais-Steider et al., 1983). Geisler & Weber (1982) have predicted the existence of such secondary structure in desmin on the basis of its entire amino acid sequence.

The CD spectrum of NF68 in 10 mM Tris buffer is distinct from the spectra of NF210 and NF160. These results are in agreement with results reported by Delacourte et al. (1982) although fractions assigned to the individual transitions for NF160 and NF210 differ considerably from the values reported here. The differences in secondary structure between the three polypeptides suggest that they have different roles in the organization of the filament.

Evidence from sequence analysis (Geisler & Weber, 1982) and proteolytic digestion studies (Geisler & Weber, 1982; Steinert et al., 1978; Steinert, 1976) indicates that intermediate filament subunit proteins contain an extensive α-helical domain of approximately 300 residues in the internal portion of the polypeptide chain. Results from CD spectroscopy of NF68 in 10 mM Tris buffer are consistent with a similar organization, since approximately half the residues appear to be organized into an α-helix. Domains of α-helices exceeding 20 residues are unstable when in direct contact with aqueous solvents (Privalov, 1982) and require lateral interactions to stabilize the helices. The highly α-helical muscle proteins tropomyosin, paramyosin, and myosin maintain their secondary structure in solution only when present as dimers and higher order aggregates (Privalov, 1982). The extensive helicity present in the 68K protein may be coincident with its existence as an oligomer in 10 mM Tris buffer.

Velocity sedimentation experiments show that in 10 mM Tris at low concentrations, 0.233 mg/mL, the major species has $s_{20,w} = 4.8$ S. Large aggregates were undetectable. The single boundary and flat plateau are insufficient criteria for homogeneity due to the highly nonideal conditions in which the experiments were performed. At higher concentrations, 1.6 mg/mL, level plateaus were not attained, indicating the presence of either reversible or irreversible self-associated molecular species. These results are similar to those of Huiatt et al. (1980) with purified desmin. This protein, a 55 000-dalton polypeptide, displays a major boundary in 0.01 M Tris of $s_{20,w} = 5.2$ S.

Interpretation of results from equilibrium sedimentation experiments is hindered by the presence of both extensive nonideality and heterogeneity. Extrapolation to zero concentration yielded $M_{w,app}$ near 210 000, suggeting that the smallest detectable species at concentrations in the range 0.05–0.20 mg/mL in 0.01 M Tris is a trimer of NF68. Model calculations suggest that the observed heterogeneity arises from a mixture of oligomeric species consisting mostly of trimer, with a small amount of one or more higher oligomers. A simultaneous contribution to the data from reversible association of chains cannot be ruled out. The overall pattern of association of NF68 remains to be elucidated.

Models of the intermediate filament protofilament consisting of three polypeptide chains aligned in register have been proposed by Steinert (1978). An alternative model, a two-chain coiled coil, has been proposed by McLachlan (1978) and by Geisler & Weber (1982). The sedimentation coefficient of NF68 would support either model, since it suggests that the conformation is extended rather than spherical. Trimers of a rodlike molecule can be made by placing the monomers end to end, side by side, or in any of a number of intermediate staggered arrangements in which the long axes of the monomers are parallel but their ends are not in register. If it is assumed that NF68 in 0.01 M Tris is solvated to the extend of 0.2 g/g (a reasonable intermediate assumption) and that the NF68 monomer is a rodlike molecule with a length of 490 Å and a diameter of 12 Å (dimensions that assume a maximum amount of α-helix), then sedimentation coefficients for various sorts of trimers can be calculated. Assuming a prolate ellipsoid, an end to end trimer would have an axial ratio of about 147; a side by side arrangement would have an axial ratio of about 20, while a staggered arrangement would have an intermediate value. Calculated sedimentation coefficients of end to end and side by side arrangements would be about 2.8 S and 6.1 S, respectively. The observed value of 4.8 S is intermediate between these values, consistent with a staggered arrangement of chains within a possible trimer of rodlike molecules. Such a staggered arrangement would be compatible with either a two-chain or a three-chain structure. We emphasize, however, that the hydrodynamic behavior may be affected by the presence of the nonhelical amino- and carboxy-terminal regions which were not considered in calculating model arrangements.

It will be desirable in the future to extend these physical studies to include buffers of higher ionic strength. Such an extension will be complicated by the likelihood that further aggregation of the proteins will occur as assembly-promoting conditions are approached. The experimental difficulties presented by low solubility and aggregation of NF68, and some of the resulting ambiguities of interpretation, might be alleviated by working with α-helical rod domains prepared by limited proteolytic digestion, in analogy to those prepared from keratin and desmin (Steinert, 1978; Geisler & Weber, 1982), if sufficient quantities of homogeneous material could be obtained.

Reassembly experiments were performed on 10 mM Tris-soluble protein to assess its capacity for forming intact intermediate filaments. It was assumed that the oligomers were directly incorporated into the polymers with no intermediate dissociation steps. The 10 mM Tris species formed short rods less than 100 nm in length and 5–20 nm in diameter. At longer reassembly times, rods were replaced with bundles of fibrous aggregates. Metal-shadowed preparations of NF68

in 10 mM Tris containing 20% glycerol revealed short rods exhibiting the characteristic 200-Å axial periodicity seen in native neurofilaments (Henderson et al., 1982; Milam & Erickson, 1982), indicating that NF68 is able to form rods exhibiting a feature of the native structure.

Although filaments are obtained from NF68 dialyzed into reassembly buffer from 10 mM Tris or directly from denaturing solutions containing urea or guanidine hydrochloride, the reassembled species differ considerably from native filaments. Isolated spinal cord neurofilaments are long smooth-walled fibers with little detectable aggregation of individual filaments. In contrast, the filaments reassembled in vitro are considerably shorter, have a greater range of diameters, display irregularities along their lengths, and are often highly aggregated. Similar results have been reported for filaments assembled in vitro from protein denatured in guanidine hydrochloride (Geisler & Weber, 1982), urea (Liem, 1982), and SDS (Zackroff et al., 1982).

The basis for the morphological variation in the reassembled filaments in unknown. CD spectra of NF68 in 10 mM Tris buffer after previous denaturation either in guanidine hydrochloride or in urea are virtually superimposable (M. R. Lifsics, unpublished results). This is in contrast to results published by Delacourte et al. (1982), who report substantial differences in the CD spectra of the 68K protein in borate buffer after previous denaturation in either urea or guanidine. Differences in results may arise from differences in the buffers employed or from incomplete removal of the denaturing agents.

Acknowledgments

We thank Kate Welch for superb technical support, Dr. John J. Correia for much helpful discussion, Drs. David Puett and James O. Gailit for assistance with CD spectroscopy, Dr. Howard E. Smith for use of the Cary 60 spectropolarimeter, and Drs. Harold Erickson and Leslie Milam for preparation and electron micrographs of the metal-shadowed specimen.

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Carbon Monoxide Binding to the Ferrous Chains of [Mn,Fe(II)] Hybrid Hemoglobins: pH Dependence of the Chain Affinity Constants Associated with Specific Hemoglobin Ligation Pathways†

Neil V. Blough‡ and Brian M. Hoffman*

ABSTRACT: In mixed-metal [Mn,Fe] hybrid hemoglobins (Hb), the two chains of a single type, α or β, are substituted with manganese protoporphyrin IX, which does not bind CO in either the Mn(II) or Mn(III) valency states. Thus, CO binding by the two ferrous subunits of a hybrid with Mn of either valency represents a simplified two-step Hb ligation process in which ligands bind to a single-chain type. Considering the [Mn(II),Fe(II)] hybrids, which are deoxy T-state analogues, at pH 6.6 both types bind CO with low affinity (α-Fe, 0.38 mmHg; β-Fe, 0.71 mmHg) and noncooperatively (Hill coefficient $n = 1$). At elevated pH, both exhibit an increase in affinity (Bohr effect) and strong cooperativity, with the α-Fe hybrid having a higher degree of cooperativity ($n \simeq 1.6$) than β-Fe ($\simeq 1.3$) at pH 9.0. The CO association constants for the Hb ligation routes in which the first two ligands bind to the same chain type are obtained from these measurements, and their pH dependence provides estimates of the proton release at each step. Through studies of CO on- and off-rates, the [Mn(III),Fe(II)] hybrids are used to obtain the pH dependence of the association constants for binding the fourth CO to the individual Hb chains. These results are used to parameterize an extended form of the two-state, allosteric model for Hb cooperativity and provide the first direct determination of the pH dependence of the CO affinity constants for the individual chains in the T and R conformations, $K_{T\alpha(\beta)}$ and $K_{R\alpha(\beta)}$, the ratios of these constants, C_α and C_β, and the concentration ratio of the low- to high-affinity structural forms of unliganded Hb, L_0. The α and β chains show a similar T-state Bohr effect; at pH 7.1, ligation of either α or β chains releases $\sim$0.4 proton. In contrast, ligation of the chains within the R state releases at most $\sim$0.2 proton at this pH. From pH 6.6 to 9.0, C_α varies $\sim$2-fold and C_β 4-fold. The analysis indicates that even the extended Monod–Wyman–Changeaux model, which accounts for chain differences, incompletely describes Hb cooperativity, since multiple values of L_0 are required to accommodate the data, and supports the suggestion of Weber regarding subunit interactions.

Hemoglobin A (Hb A) cooperatively binds four ligands via a complex sequence of intermediate liganded states. Valency and NO hybrid hemoglobins have been used to model the behavior of Hb intermediates in the latter stages of the ligation process (Shulman et al., 1975; Baldwin, 1975; Szabo & Karplus, 1975). In this and in the following paper (Blough et al., 1984), metal substitution in the form of mixed-metal [Mn,Fe][1] hybrids (Hoffman, 1979; Waterman & Yonetani, 1970; Gibson et al., 1974; Hoffman et al., 1975; Blough & Hoffman, 1982) is shown to offer a particularly advantageous and direct means with which to probe both initial and final stages of CO binding by Hb. In combination with our earlier work, the results presented here comprise the first self-consistent set of CO binding parameters for individual chains within the two major quaternary states of hemoglobin. The pH response of CO binding to the hybrids further permits us to estimate the proton release associated with individual stages in the ligation process, as well as the tertiary Bohr effect associated with ligation of an individual chain within the T or the R quaternary structure.

In [Mn,Fe] hybrids, two chains of a single type, α or β, are substituted with manganese protoporphyrin IX, and the two complementary chains are retained in the ferrous form. The Mn-containing subunits can exhibit either the Mn(II) or Mn(III) valency states, and in neither case do they bind CO. Thus, ligand binding by the two ferrous subunits of a hybrid with Mn of either valency represents a simplified Hb ligation process in which ligands bind to a single-chain type. The early steps of Hb ligation are investigated with the [Mn(II),Fe(II)] hybrids. Work to date has established the following: fully reconstituted Mn(II) Hb is functionally equivalent to native Hb A (Gibson et al., 1974; Hoffman et al., 1975; Gibson & Hoffman, 1979); [Mn(II),Fe(II)] hybrids are functional analogues of unliganded, T-state Hb A (Blough et al., 1980; Blough & Hoffman, 1982). This was expected because of the

† From the Department of Chemistry and the Department of Biochemistry, Molecular and Cell Biology, Northwestern University, Evanston, Illinois 60201. *Received June 14, 1983; revised manuscript received January 12, 1984.* This work was supported by Grant HL 13531 from the National Institutes of Health.

* Address correspondence to this author at the Department of Chemistry.
‡ Present address: Laboratory of Chemical Biodynamics, University of California, Berkeley, CA 94720.

[1] Abbreviations: Hb, hemoglobin; met-Hb, ferric form of Hb; [Mn,Fe], hemoglobin derivative in which the two chains of a single type, α or β, are substituted with manganese protoporphyrin IX; Bis-Tris-HCl, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane hydrochloride; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; MWC, Monod–Wyman–Changeaux model of cooperative ligand binding.

0006-2960/84/0423-2875$01.50/0