Biochem. J. (1989) 264, 53–60 (Printed in Great Britain)

Characterization of two proteolytically derived soluble polypeptides from the neurofilament triplet components NFM and NFH

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We have purified to homogeneity the regions derived by chymotryptic digestion of the ox neurofilament polypeptides NFH and NFM; the regions, called M_1 and M_2, are thought to form part of the projecting sidearms of mammalian neurofilaments [Chin, Eagles & Maggs (1983) Biochem. J. **215**, 239–252]. They were isolated and purified under non-denaturing conditions and showed no tendency to interact with each other in solution. The M_r values obtained by sedimentation are approx. 61000 for M_1 and 42000 for M_2, considerably lower than the values obtained by SDS/polyacrylamide-gel electrophoresis. These M_r values were unchanged in the presence of 6 M-guanidine hydrochloride, suggesting that the regions exist as monomers in solution. Both M_1 and M_2 are highly phosphorylated, and there is only a slight change in the sedimentation value upon dephosphorylation. Dephosphorylation of M_1 with alkaline phosphatase was more than 90% efficient but was never absolute. Dephosphorylation of M_2 was complete. Both M_1 and M_2 bind Ca^{2+}; in the case of M_1, this binding is phosphorylation-dependent. M_1 also binds cytochrome *c*, and dephosphorylation affects binding. In similar conditions, neurofilaments bind at least twice their own mass of cytochrome *c*, owing to their opposite net charges. No interactions were observed between native or dephosphorylated M_1 and M_2, and intact neurofilaments under a wide variety of conditions. These results are discussed in terms of the possible roles that neurofilament sidearms might play and throw doubt upon their supposed function of rigidly cross-linking neurofilaments together within the axoplasm of neurons.

INTRODUCTION

Previous studies from this laboratory (Chin *et al.*, 1983; Eagles *et al.*, 1985) and elsewhere (Julien & Mushynski, 1983; Carden *et al.*, 1985) have demonstrated that proteolytic degradation of mammalian neurofilaments can be used to help define domains within the constituent polypeptides. Digestion of ox neurofilaments with trypsin yields a complicated pattern of breakdown products, but analysis indicates that large fractions of NF160 (NFM) and NF200 (NFH) can be removed while still maintaining structurally discernible 10 nm filaments which can be seen under the electron microscope (Chin *et al.*, 1983). These removable regions are thought to be located peripherally to the filament core and are anchored therein by the rest of the polypeptide (for review, see Eagles *et al.*, 1989). Digestion with chymotrypsin of the NFM and NFH polypeptides present in intact filaments results in the rapid disruption of the filament backbone and the appearance of two major proteins with apparent M_r values in SDS/polyacrylamide-gel electrophoresis (PAGE) of 120000 and 170000. The M_r-120000 protein (M_2) is derived from NFM and shows those tryptic peptides which are thought to be peripherally localized; it is phosphorylated (Carden *et al.*, 1985). The M_r-170000 protein (M_1) comes from NFH. It is also phosphorylated (Carden *et al.*, 1985) and is most probably peripherally arranged. It has been argued that M_1 and M_2 are strong candidates for being parts of the structures that extend from the neurofilament backbone and appear to bridge neighbouring filaments in intact neurones (Chin *et al.*, 1983; Eagles *et al.*, 1985; Eagles, 1986).

In this paper we present our findings on the characterization of these proteins, and we discuss their potential for acting as structures that might connect filaments together.

MATERIALS AND METHODS

Preparation of M_1 and M_2

About 90 ml of purified ox neurofilaments (1–2 mg/ml) (prepared as in Chin *et al.*, 1983) were spun at 100000 *g* at 10 °C for 2 h. The supernatant was discarded and the pellet containing neurofilaments was resuspended in 45 ml of 0.1 M-NaCl/10 mM-sodium phosphate/0.02% NaN_3, pH 7.0, and dialysed against the same buffer overnight. The dialysed sample was digested with α-chymotrypsin using similar conditions as described previously (Chin *et al.*, 1983), and the supernatant containing the polypeptides M_1 and M_2 was dialysed against 50 mM-imidazole/HCl/0.02% NaN_3, pH 7.0 (Buffer I). The dialysed sample was applied to a column (19.5 cm × 1.5 cm) packed with DEAE-52-cellulose (Whatman) that had been pre-equilibrated with Buffer I. The sample was eluted as follows: (i) a gradient composed of 100 ml of Buffer I and 100 ml of Buffer I containing 0.2 M-NaCl, (ii) 100 ml of buffer I containing 0.2 M-NaCl,

Abbreviations used: PAGE, polyacrylamide-gel electrophoresis; DTT, dithiothreitol.

and (iii) a gradient composed of 100 ml of Buffer I containing 0.2 M-NaCl and 100 ml of Buffer I containing 0.3 M-NaCl.

Fractions (5.3 ml each) were collected and analysed by SDS/PAGE. Those containing pure M_1 and M_2 were pooled and dialysed against Buffer I. After dialysis, the pooled samples were concentrated by applying them to a small column of DEAE-52-cellulose (0.5 ml total volume) that had been pre-equilibrated with the same buffer. The sample (M_1 or M_2) was then eluted with Buffer I containing 0.5 M-NaCl. Fractions of 0.5 or 1.0 ml were collected and dialysed against Buffer I. Unless stated otherwise, all operations were performed at 4 °C.

Determination of Stokes radii of M_1 and M_2 using Sephacryl S-300

A column (96 cm × 1 cm) of Sephacryl S-300 (Pharmacia) was employed for the determination of Stokes radii. The column was equilibrated with 20 mM-Tris/HCl/130 mM-KCl/0.1 mM-dithiothreitol (DTT)/0.02% NaN_3, pH 7.4, before samples were applied. Standard samples (thyroglobulin, alcohol dehydrogenase, catalase, urease, albumin, ferritin, aldolase and xanthine oxidase), and samples of M_1 and M_2 (about 1–5 mg of each) were dialysed and dissolved in minimal amounts of this buffer. These samples were applied to the column, which was run at room temperature, and fractions of 1.35 ml were collected. Analysis was by SDS/PAGE. Calculations were performed according to the method of Horiike *et al.* (1983); values for the Stokes radii of standard proteins were also taken from this reference.

Estimation of M_r using Sephacryl S-400

Estimation of M_r values in 6 M-guanidine-hydrochloride was performed using a Sephacryl S-400 (Pharmacia) column (95 cm × 1 cm). The column was equilibrated at room temperature with 6 M-guanidine hydrochloride/0.02 M-Tris/HCl/0.1 mM-DTT, pH 7.0 (Buffer G). Standard proteins of known M_r (myosin, phosphorylase *b*, bovine serum albumin, catalase, aldolase, creatine kinase, glyceraldehyde 3-phosphate dehydrogenase, lysozyme, actin) and other samples (M_1, M_2, *Myxicola* neurofilaments) were dissolved in and dialysed against Buffer G, and boiled. Ox filaments were precipitated with 70% (v/v) ethanol, dried, dissolved in Buffer G and boiled before applying them to the column. Fractions (1 ml) were collected at room temperature and analysed by SDS/PAGE.

Determination of sedimentation coefficients

A Spinco model E analytical ultracentrifuge was used with an RTIC temperature measurement system. Sedimentation coefficients were corrected to standard conditions (Tanford, 1961).

Sedimentation equilibrium measurements

A Beckman Model E analytical ultracentrifuge was used. The 'intermediate speed' method was employed (Creeth & Harding, 1982). Values for partial specific volumes ($\bar{v}$) were 0.739 ml·g^{-1} for M_1 and 0.731 ml·g^{-1} for M_2. These values were calculated using the Traube rule (see Cohn & Edsall, 1943) from the amino acid composition data of Weber and coworkers (M_1, Geisler *et al.*, 1985; M_2, Geisler *et al.*, 1984, excluding values for tryptophan and cysteine, which were not determined).

Whole-cell weight average relative molecular masses, $M^0_{r,w}$, and point weight average relative molecular masses, $M_{r,w}$, were obtained as previously described (Creeth & Harding, 1982).

Determination of phosphate content

Samples to be assayed were extensively dialysed against distilled water. To solubilize filaments, 0.1 M-NaOH was used. Fractions containing not more than 20 μg of protein were ashed in acid-washed tubes for 30 min, at 210 °C with 25 μl of 70% $HClO_4$. After ashing, samples were cooled and 1 ml of 1.2 M-HCl was added, followed by 0.33 ml of P_i reagent (1 vol. of 10% ammonium molybdate in 4 M-HCl added to 3 vol. of 0.2% Malachite Green). After 5 min, the developed colour was measured at 660 nm in 1 cm semimicro cuvettes. Phosphate standards ranging from 2 to 10 nmol of potassium phosphate were also ashed and treated in the same way.

Determination of nitrogen content

For nitrogen determinations, samples were ashed as described above after first being dialysed exhaustively against distilled water. Following ashing, 0.5 ml of distilled water was added and mixed together with 0.5 ml of phenol reagent (5 g of phenol and 0.025 g of sodium nitroprusside in 250 ml of water). Then, 0.2 ml of hypochlorite reagent (33.8 mM-sodium hypochlorite in 2.5 M-NaOH) was added. After 20 min, the absorbance of the developed colour was measured at 578 nm. Standards were prepared from ammonium sulphate. Protein concentrations were calculated assuming 16% (w/w) nitrogen. All glassware was thoroughly cleaned by soaking in 50% H_2SO_4 for 2–3 days with several changes of acid.

Dephosphorylation

Escherichia coli alkaline phosphatase (type III from Sigma) was used for dephosphorylation (Carden *et al.*, 1985). The alkaline phosphatase was dialysed (in 50 mM-Tris/HCl/100 mM-NaCl/1 mM-$ZnCl_2$/0.02% NaN_3, pH 7.5 or pH 9.5) overnight at 37 °C together with the sample after prior treatment with phenylmethanesulphonyl fluoride, Tos-Lys-CH_2Cl (TLCK; 7-amino-1-chloro-3-L-tosylamidoheptan-2-one) and Tos-Phe-CH_2Cl (TPCK; 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one), all at 1 μg·ml^{-1}. At least 1 unit of alkaline phosphatase was used for every 0.2 mg of protein.

Binding of cytochrome *c*

Interaction of native ox neurofilaments (0.5–2.0 mg·ml^{-1}) with cytochrome *c* was performed routinely at room temperature in 20 mM-histidine monohydrochloride (Sigma)/0.1 M-NaCl/0.02% NaN_3, pH 7.0 (NaOH). NaCl was always included to prevent filament dissociation. The mixture was spun at room temperature in a Beckman Airfuge (172 kPa, 120000 ***g*** for 1–3 h) with or without a sucrose pad (20% sucrose in the same buffer). After centrifugation, the pellets were washed with the buffer before preparing them as samples for SDS/PAGE; standard amounts of cytochrome *c* (1–3 μg) were also run on the same gel. After staining with Coomassie Blue, the gel was analysed by densitometry and the standards were used to compute a value for the change in absorbance/μg of cytochrome *c* ($\Delta A/\mu g$). This value was then used to determine the amount of cytochrome *c* (μg) in a particular sample. Plots of cytochrome *c* bound to fixed amounts of neurofilaments

versus total cytochrome *c* yielded hyperbolic curves showing saturable binding. The maximum binding capacity of samples was estimated by extrapolation from the linear portion of the graph. Interactions between cytochrome *c* and native and dephosphorylated M_1 and M_2 were carried out using a Sephacryl S-300 column (26 cm × 1.2 cm) which was equilibrated with 20 mM-histidine monohydrochloride/0.1 M-NaCl/0.02% NaN_3, pH 7.0 (NaOH) containing 0.01 mg of cytochrome $c \cdot ml^{-1}$. Incubation mixtures in buffer containing cytochrome *c* and 1–2 mg of M_1 or M_2 were applied to the column and eluted at room temperature. Fractions (1.2 ml) were collected and analysed by SDS/PAGE.

Binding of Ca^{2+}

To detect the binding of Ca^{2+} to M_1 and M_2, SDS/PAGE was performed on column-purified samples, and the proteins were transferred to nitrocellulose by blotting under pressure for around 12 h at 4 °C. The nitrocellulose membranes were then briefly soaked in distilled water and incubated with ^{45}Ca (Amersham) for 1–2 h at room temperature. After washing, labelled proteins were identified by autoradiography.

Protein determination and electron microscopy

These techniques were performed as previously described (Chin *et al.*, 1983).

RESULTS

Due to the nature of our work, we aimed to recover as much of the *C*-terminal domains of NFH and NFM as possible, so digestion times were kept to a minimum in the hope that only the major site of proteolytic cleavage, near to the rod region, would be cleaved. Separation of proteolytic digests on DEAE-cellulose (Fig. 1*a*) shows this to be the case, though clearly some fractions contain many minor polypeptides of lower M_r than the major products, indicating further cleavage at other sites. For subsequent work, fractions containing only the single high-M_r cleavage products of NFM and NFH were pooled and concentrated. Samples of these proteins are shown in Fig. 1(*b*). At no time during the purification of M_1 and M_2 were samples subjected to conditions likely to engender denaturation or loss of potential binding capacity.

Table 1. M_r value of neurofilament proteins M_1 and M_2 in non-dissociating (A) and dissociating (B) solvents, determined by low-speed sedimentation equilibrium

Solvent A, 10 mM sodium phosphate/0.1 M-NaCl/0.02% NaN_3, pH 7.0; solvent B, solvent A + 6 M-guanidine hydrochloride. $M^0_{r,w}$ is the weight average relative molecular mass over the whole-cell solute distribution; whereas $M_{r,w}$ ($J \rightarrow 0$) is the point weight average relative molecular mass extrapolated to zero (fringe) concentration.

Protein	Solvent	$M^0_{r,w}$	$M_{r,w}(J \rightarrow 0)$
M_1	A	61000 ± 2000	60000 ± 5000
M_1	B	64000 ± 2000	60000 ± 5000
M_2	A	41500 ± 1500	42000 ± 4000
M_2	B	44000 ± 1500	40000 ± 4000

Table 1 presents values obtained for the M_r values of M_1 and M_2 from 'intermediate' speed (Creeth & Harding, 1982) sedimentation equilibrium experiments. Two types of M_r are presented. The weight average relative molecular mass for the whole solute distribution, $M^0_{r,w}$ is obtained by extrapolation of the M^* function to the cell base. Despite the low cell-loading concentrations used, these values might be affected by the presence of any thermodynamic non-ideality or self-association phenomena. Therefore we have also included in Table 1 values of the point weight average relative molecular mass ($M_{r,w}$) extrapolated to zero (fringe) concentration.

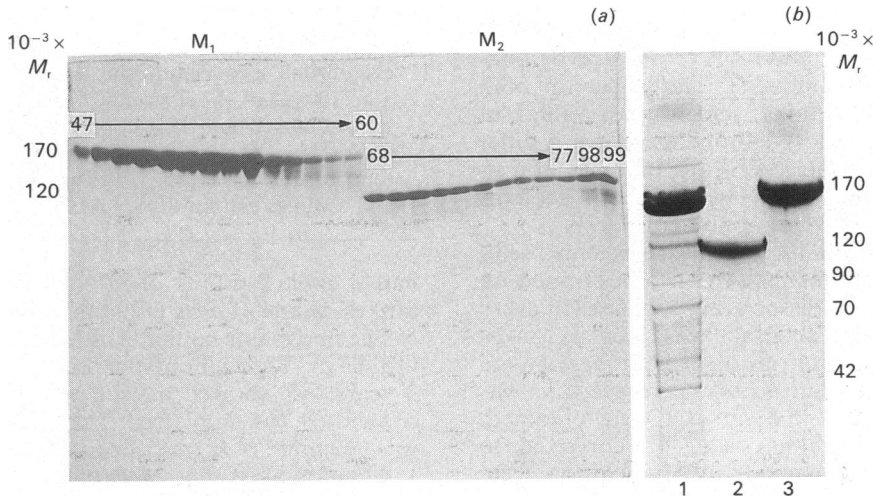

Fig. 1. Separation of proteolytic digests of neurofilament proteins

(*a*) Analysis by SDS/PAGE (5–20% gradient) of column fractions obtained after supplying chymotryptic digest to column packed with DEAE-cellulose. The elution protocol was as described in the Materials and methods section. The regions containing M_1 and M_2 are marked and the fraction numbers are also given. (*b*) SDS/PAGE of samples of M_2 (lane 2) and M_1 (lane 3) that were used for all investigations. Lane 1 shows a sample of *Myxicola* neurofilaments for comparison of M_r in this system.

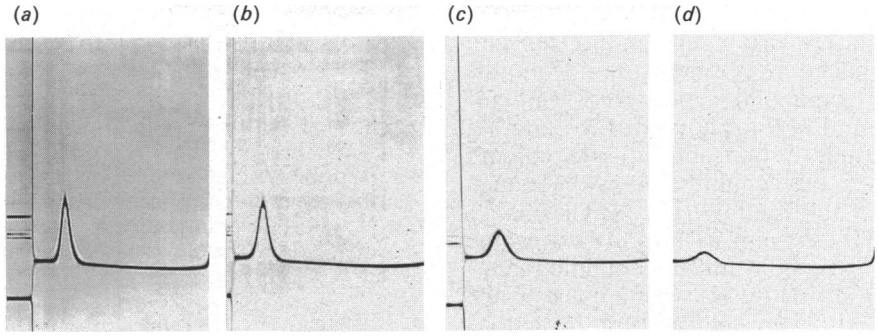

Fig. 2. Sedimentation diagrams of phosphorylated and dephosphorylated forms of M_1 and M_2

The buffer used was 10 mM-sodium phosphate/0.1 M-NaCl/0.02% NaN_3, pH 7.0. In all cases, sedimentation was at 59780 rev./min in a 12 mm double sector cell. (*a*) M_1: 3.25 mg·ml^{-1}; 19.0 °C; bar angle 70°. (*b*) Dephosphorylated M_1: 3.08 mg·ml^{-1}; 21.5 °C; bar angle 70°. (*c*) M_2: 1.72 mg·ml^{-1}; 23.5 °C; bar angle 65°. (*d*) Dephosphorylated M_2: 1.07 mg·ml^{-1}; 22.0 °C; bar angle 70°.

Table 2. Phosphate contents of M_1, M_2 and neurofilaments

Each determination was carried out in triplicate. The numbers of separate preparations (*n*) used to calculate the means are given.

Protein	P_i (nmol/mg of protein)
Ox neurofilaments	269±52 (n = 8)
Ox neurofilaments after treatment with alkaline phosphatase	95–177 (pH 7.5) 62–71 (pH 9.5)
M_1	949±264 (n = 7)
M_1 treated with alkaline phosphatase	75±4 (pH 9.5)
M_2	516±36 (n = 5)
M_2 treated with alkaline phosphatase	Not detected
Myxicola neurofilaments	166±10 (n = 3)
Myxicola neurofilaments treated with alkaline phosphatase	Not detected

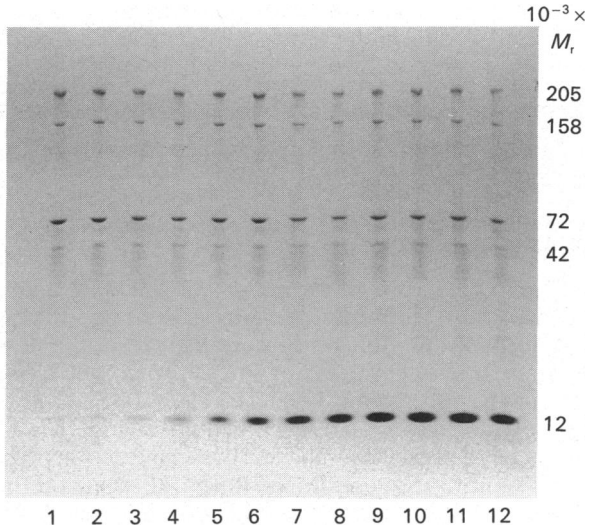

Fig. 3. Analysis by SDS/PAGE (5–15% gradient) of the interaction between cytochrome *c* and native ox neurofilaments

Neurofilaments (1.5 mg·ml^{-1}) were incubated with cytochrome *c* concentrations (lanes 1–12) of 0, 0.2, 0.5, 1, 3, 6, 10, 12, 14, 17, 19, and 21 mg·ml^{-1}. Samples (100 µl) were removed and laid upon a 50 µl sucrose pad (20% sucrose in the same buffer) in an Airfuge tube. After centrifugation (3 h), the pellet was washed briefly in buffer and samples run on SDS/PAGE.

Although these values cannot be as precisely determined as the $M^0_{r,w}$ values, they should not be affected by non-ideality or associative phenomena. The values for both proteins in a non-dissociating solvent (M_r values of around 60000 for M_1 and 42000 for M_2) did not differ appreciably from those obtained in the presence of 6 M-guanidine hydrochloride, implying that both proteins exist in solution essentially as monomeric forms.

In addition to the equilibrium measurements, sedimentation velocity experiments were also performed on native M_1 and M_2 in non-dissociating solvents. In every case, a single symmetrical peak was seen (Fig. 2). Although this is not in itself proof of homogeneity, no evidence for any dissociation or polydispersity was observed. Linear plots of ln r versus t were obtained. Values of the sedimentation coefficient, corrected to water at 20 °C and extrapolated to infinite dilution, were 2.45×10^{-13} s for native M_1 protein and 2.1×10^{-13} s for native M_2. These values were slightly lower for samples that had been dephosphorylated (2.3×10^{-13} s for M_1 and 2.01×10^{-13} s for M_2). Thus gross structural changes are unlikely to accompany phosphate removal.

Estimates for the Stokes radii of M_1 and M_2 were obtained using the methods of Horiike *et al.* (1983). M_1 had a Stokes radius of 60Å (600 nm) and M_2 had a Stokes radius of 52Å (520 nm).

The phosphate contents of M_1 and M_2 were measured. (Table 2). Throughout the study, which extended over 3 years, M_1 showed a wide variation in its phosphate content (±264 nmol/mg). The minimum value obtained was 619 nmol of P_i/mg of protein and the maximum was 1340 nmol of P_i/mg of protein. The variation in the phosphate content of M_2 was much less (±36 nmol/mg). Dephosphorylation was fully effected for M_2 and for *Myxicola* neurofilaments. Residual phosphate, unremovable by the conditions used, was always detected with whole ox neurofilaments and M_1. Thus the sites on these molecules clearly differ in the ease with which they yield phosphate.

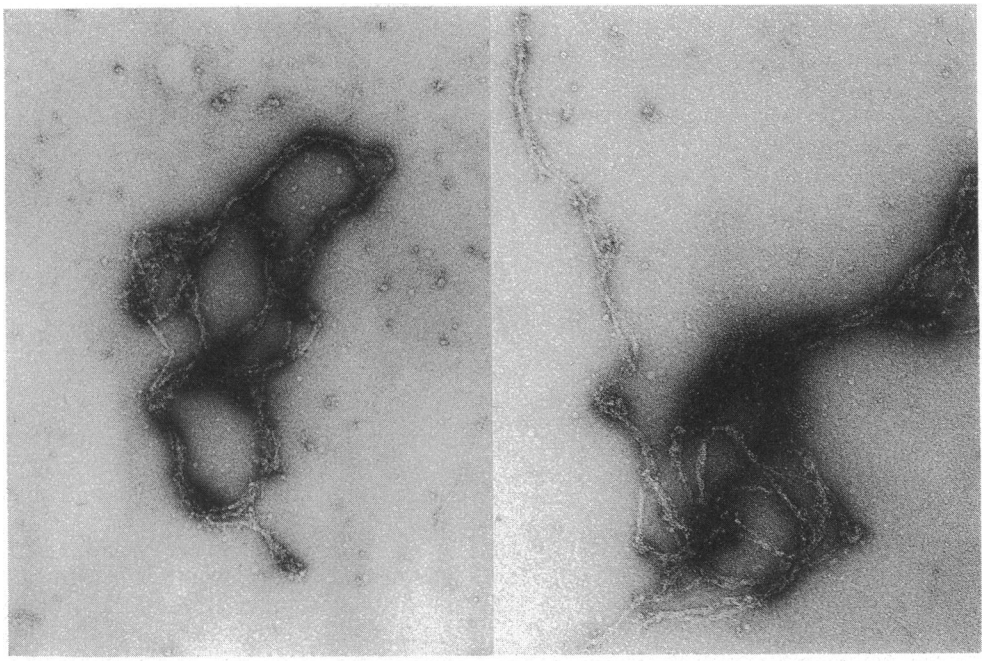

Fig. 4. Electron micrographs of neurofilaments that have been incubated with cytochrome *c*

Conditions were similar to those described in Fig. 3. A loose association of filaments is seen, with filaments interacting mainly along their lengths. Magnification ×75000.

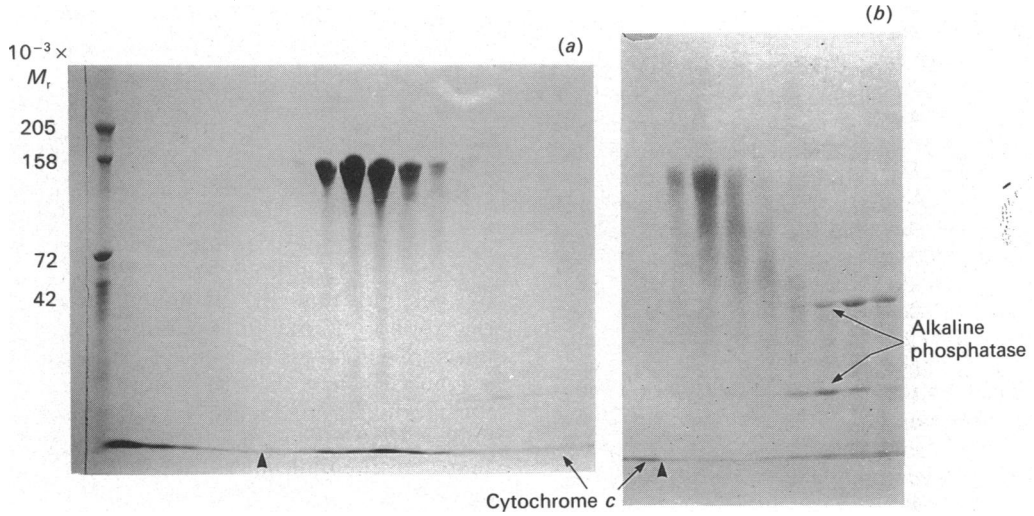

Fig. 5. Analysis by SDS/PAGE of M_1 from fractions eluted from a Sephacryl S-300 column

(*a*) Untreated M_1, (*b*) dephosphorylated M_1. Corresponding regions of the column profile are shown here with the positions of cytochrome *c* and alkaline phosphatase marked. The arrowheads on (*a*) and (*b*) show the background levels of cytochrome *c*. Standards are included in some of the other lanes.

To assess the possibility that M_1 or M_2 might be involved in connecting neurofilaments together, we investigated their capacity for binding to native neurofilaments under various conditions including (1) using phosphorylated and dephosphorylated preparations of M_1, M_2 and neurofilaments, (2) varying the buffers (Mes, Pipes, Tris, phosphate), ionic strength, nucleotides (ATP, GTP), Ca^{2+} and Mg^{2+} or (3) adding additional proteins, namely tubulin, taxol-stabilized microtubules, actin and phalloidin-stabilized actin filaments. Under a wide variety of experimental conditions and arrangements, no consistent interactions were observed when either M_1 or M_2 were admixed with filaments and then spun on an Airfuge and the pellets assayed for the binding of these two proteins to the sedimented neurofilaments. Ca^{2+} in the millimolar range increased the susceptibility with which filaments sedimented.

However, an interaction was demonstrated between cytochrome *c* and neurofilaments. The reason for investigating this system was that Gilbert *et al.* (1975) had previously observed binding of cytochrome *c* to

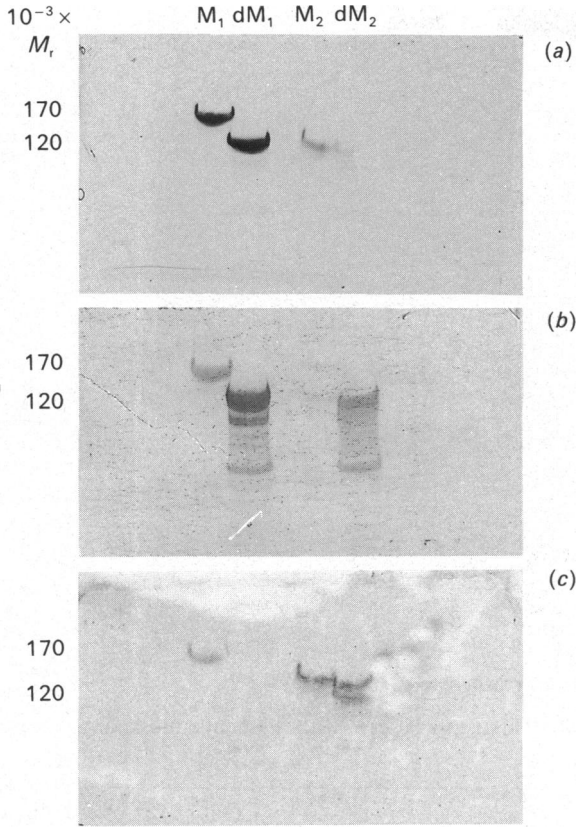

Fig. 6. Interactions of M_1 and M_2 with Ca^{2+}

Samples of M_1 and M_2 before (M_1 and M_2) and after (dM_1, dM_2) treatment with alkaline phosphatase were run on a gradient gel (SDS/PAGE, 5–15%). The proteins were then transferred to nitrocellulose membrane which was washed with ^{45}Ca. After rinsing, the membrane was subjected to autoradiography. (*a*) Gel after transfer of protein to the membrane. The gel was stained with Coomassie Blue. (*b*) Nitrocellulose membrane after staining for protein with Amido Black. Dephosphorylation of M_1 results in an increased mobility in SDS/PAGE. A small increase in mobility is observed with M_2 on dephosphorylation. M_2 transfers less well in the phosphorylated than in the dephosphorylated form. The extra bands in dM_1 and dM_2 arise from the alkaline phosphatase. (*c*) Autoradiograph. M_1 binds ^{45}Ca unless it is first dephosphorylated. M_2 binds ^{45}Ca in both its phosphorylated and dephosphorylated forms.

Myxicola neurofilaments, an event which promotes the bundling of filaments in close association and the formation of neurofilament rings. We reasoned that a study of the binding of cytochrome *c* to ox neurofilaments might throw light on factors influencing the attachment of proteins to neurofilaments and cross-bridge formation.

Fig. 3 shows the results of an experiment designed to measure the binding capacity of ox filaments for cytochrome *c*. Densitometry of gels such as this shows that native neurofilaments in 20 mM-histidine/0.1 M-NaCl/0.02% NaN_3, pH 7.0, can bind a maximum of about twice their weight of cytochrome *c* [$2.18\pm0.03\,\mu g$ of cytochrome *c* was bound to 1 μg of protein ($n = 3$)]. Increasing the NaCl concn. to 0.3 M abolishes this. The presence of phosphate affects binding, with a reduction of 60% being seen on going from 10 mM-phosphate to 50 mM-phosphate in the buffer. Treatment of filaments with alkaline phosphatase did not change by very much the maximum amount of cytochrome *c* that could be bound. After dephosphorylation, which was never seen to be total, the binding capacity was $2.05\pm0.1\,\mu g$ of cytochrome $c\cdot\mu g$ of protein^{-1} ($n = 5$). Electron microscopy of filaments treated with cytochrome *c* showed that they became bundled. Ring-like structures were also seen (Fig. 4).

The phosphorylated regions M_1 and M_2 also bind cytochrome *c*, though the interaction is weak. Binding was detected by using a Sephacryl S-300 column with cytochrome *c* in the running buffer. Fig. 5 shows the results with phosphorylated M_1 and with a dephosphorylated sample. It is clear that M_1 binds cytochrome *c*, as they elute together. In the dephosphorylated sample binding is much reduced, the cytochrome *c* level in the region at which M_1 elutes being little above background.

Fig. 6 shows the results of an experiment designed to test whether Ca^{2+} can interact with M_1 or M_2 and, if so, whether phosphorylation of the protein is important. M_1 binds Ca^{2+}, but not if it is first dephosphorylated. M_2 also binds Ca^{2+}, although under the conditions used binding is unperturbed by alkaline phosphatase treatment.

DISCUSSION

We report here the purification and properties of the *C*-terminal domains of NFH and NFM following their removal by digestion with chymotrypsin.

The M_r of the chymotryptic fragment M_1, as determined by this work, is approx. 61000 and that for M_2 is around 42000. On SDS gels these fragments have apparent M_r values of approx. 170000 and 120000 respectively. These regions are therefore responsible in large part for the anomalous behaviour of the neurofilament polypeptides previously observed in the SDS system (Kaufman *et al.*, 1984). M_r determination by gel filtration in guanidine hydrochloride or by electrophoretic methods (Chin *et al.*, 1983) also seems unreliable for these molecules, as the methods depend on a comparison with standard proteins of similar conformation. In our hands, gel filtration gave values 50% higher than the values found by sedimentation. The shapes of the molecules, together with their high charge density, doubtless play a significant role in their anomalous behaviour. Shape factors have also caused discrepancies in the determination of the M_r of kinesin, a long, thin molecule (Vale *et al.*, 1985; Kuznetsov *et al.*, 1988; Bloom *et al.*, 1988).

The sedimentation values together with the M_r values for these polypeptides indicate that the proteins are probably extended in solution. If we assume a typical value for the solvation, or equivalently the swelling ratio $V_s/\bar{v}$ (where V_s is the swollen specific volume), of 1.4 (Harding & Rowe, 1982), the values for the sedimentation coefficients correspond to axial ratios of approx. 23 (M_1) and 18 (M_2) for models of equivalent prolate ellipsoids of revolution. Not dissimilar values for the axial ratios can be derived from the dimensions obtained by shadowing these regions (Eagles *et al.*, 1985) after account is taken of the thickness of the metal coat. On this basis the proteins could form the projections seen on shadowed neurofilaments (Ip, 1986; Hisanaga & Hirokawa, 1988).

Our previous studies showed that M_1 and M_2 could be easily separated under mild non-denaturing conditions (Chin *et al.*, 1983). We have shown here that these regions behave in solution as monomeric molecules. It is generally assumed that in the backbone of the neurofilament the polypeptides, including NFM and NFH, are arranged as coiled coils. Our results imply that on leaving the neurofilament core these polypeptides are not associated in stable polymeric complexes such as exist within the backbone.

The chymotryptic region M_2 can be localized within the intact molecule now that sequence data are available (Myers *et al.*, 1987; Levy *et al.*, 1987). Geisler *et al.* (1987) have shown recently that the isolated polypeptide of NFM has a major chymotryptic cleavage site around Phe-452. If the conformation of the polypeptide in the native neurofilament does not hinder cleavage, then M_2 would also be produced by action at this residue. Phe residues also exist around this position in NFH (Lees *et al.*, 1988; Dautigny *et al.*, 1988), so chymotrypsin probably cleaves near to this residue in both of the intact neurofilament polypeptides.

Much current work has focused on the aspect of differential phosphorylation within perikarya and axons (Sternberger & Sternberger, 1983; Carden *et al.*, 1987; Black & Lee, 1988). Our preparation of M_1 and M_2 is derived from neurofilaments extracted from spinal nerve roots (Carden & Eagles, 1983), a well-defined source, with little contamination from nerve cell bodies and other tissue. The values we have obtained for phosphate content therefore represent those that exist in axons. If we assume that the numbers of phosphorylation sites on these molecules are similar to those proposed for NFH and NFM in other species (Myers *et al.*, 1987; Levy *et al.*, 1987; Lees *et al.*, 1988; Dautigny *et al.*, 1988; Breen *et al.*, 1988), then in nerve root axons these proteins could be almost fully phosphorylated.

Neurofilaments bind about twice their weight of cytochrome *c* in 0.1 M-NaCl, in which the filaments are stable. This binding is almost entirely electrostatic in nature, cytochrome *c* having a strongly positive net charge at physiological pH. It has been suggested that both the polypeptides M_1 and M_2 lie away from the filament's backbone, with their anchoring domains within the core (Chin *et al.*, 1983). This arrangement would make the filaments' surface a highly charged environment (Eagles, 1986; Geisler *et al.*, 1983). Presumably it is to these *C*-terminal regions that binding of cytochrome *c* occurs, being dependent in part on the phosphorylation sites present there. This view is supported by the observation that the phosphorylation state of M_1 affects the binding of cytochrome *c* to this region.

Cytochrome *c* must also attach to other sites. We know this because dephosphorylation of ox neurofilaments, although not complete, has little effect on the maximum binding capacity for this protein. Further, dephosphorylation of *Myxicola* neurofilaments, a process which does go to completion, does not prevent cytochrome *c* binding to the filaments and aggregation of these filaments into rings. Thus the process of shielding phosphorylation sites is not the only factor contributing to filament bundling in the presence of cytochrome *c*.

Ca^{2+} also binds to the M_1 and M_2 regions. Ca^{2+} binding to neurofilaments was first reported by using *Myxicola* preparations (Abercrombie *et al.*, 1986) and has subsequently been found in mammalian (Lefebvre & Mushynski, 1987, 1988) and squid (Krinks *et al.*, 1988) preparations, although its physiological significance, if any, is uncertain. We have shown that binding of Ca^{2+} clearly involves the phosphate groups on the M_1 molecule, though for M_2 it seems that other factors, apart from the degree of phosphorylation, also affect binding.

In an earlier paper (Chin *et al.*, 1983) we suggested tentatively that both M_1 and M_2 could be candidates for forming all or part of the side-arms that are seen apparently linking neurofilaments in intact neurons. Our results here demonstrate that no strong interactions can be detected between M_1, M_2 and neurofilaments; thus it is unlikely that M_1 or M_2 on their own bind to neighbouring filaments forming a robust or rigid network. Their contribution to the surface charge on neurofilaments in solution, which overall is negative at physiological pH, causes neurofilaments to behave essentially as non-interactive components, a situation which would also exist in the high ionic strength environment of axoplasm. This architectural design would enable the neurofilament lattice to be readily dynamic, as discussed previously (Carden *et al.*, 1987; Matus, 1988). The side-arms, rather than holding the filaments together, may be preventing the close approach of filaments within neurons, and may function more like spacers, keeping neurofilaments apart.

We thank the Medical Research Council, The Wellcome Trust and The Foundation for Age Research for support. We also thank Mrs. R. Rao for excellent technical assistance, and Zolly Gabor and Chris Flood for photography. Taxol was generously provided by Dr. M. Suffness.

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Received 10 March 1989/13 June 1989; accepted 26 June 1989